Host Involvement In Nodule Initiation In The Soybean-*Bradyrhizobium* Symbiosis

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Declaration

The research in this thesis is my own work, except where acknowledgement is made. It has not been submitted for any other degree.

Anne Mathews

(ANNE MATHEWS)

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ABSTRACT

The genetics and ontogeny of nodule initiation was investigated in soybean cultivar Bragg and its mutants which are defective in nodulation. Mutants nod49, nod772 and nod139 are non-nodulating, whereas nts382 is an extreme supernodulation mutant with enhanced nodulation in the presence of nitrate. The intermediate supernodulator nts1116 and the naturally-occurring variant rj_1 were included in some of the experiments.

Complementation tests conducted on the mutants indicated that *nod*49 and *nod*772 are allelic to rj_1, while *nod*139 is not allelic to rj_1 and represents a separate complementation group. The gene symbol rj_6 is tentatively proposed for this new gene conditioning non-nodulation in nod139. Mutant nts382 represents a separate complementation group. Backcrosses to the parent cv. Bragg indicated a monogenic recessive inheritance of all the induced mutants (nod49, nod772, nod139 and nts382).

The non-nodulation mutants are similar to the wild type in the root exudates and no differences were detectable. Rhizosphere population studies indicated that the non-nodulation mutants are similar to the wild type. Mutant nts382 supported a substantially larger number of *Bradyrhizobium* in the rhizosphere while nts1116 had intermediate levels of rhizosphere colonization. All the mutants were similar to the wild type in attachment of *B. japonicum* strain USDA110. The non-nodulation mutants are blocked at an early sub-epidermal cell division stage. The total number of sub-epidermal cell divisions in mutants nod49, nod772 and rj_1(Lee) was considerably reduced but they did have a few stage I and stage II divisions. On the other hand, nod139 had no sub-epidermal cell divisions. Autoregulation which blocks the success of infections into nodules acts at about stage IV of sub-epidermal cell divisions in the wild type. This mechanism is defective in nts382. Mutants nod49, nod772 and rj_1 (Lee) lacked curled root hairs when inoculated with *B. japonicum* strain USDA110. Mutant nod772 showed occasional root hair curling which in part reflected its slight leakiness for nodulation. All the non-nodulation mutants occasionally formed nodules, and nodulation was more frequent at higher cell numbers of rhizobia. These nodules were normal in morphology and nitrogenase activity. This indicates that the blockage in these mutants was restricted to the early stage of nodule initiation.

Non-nodulation is controlled by the root whereas supernodulation is controlled by the shoot. The genetic interaction between the supernodulation mutant nts382 and the non-nodulation mutants resulted in the identification of double recessive non-nodulating mutants which demonstrate that the non-nodulation root is epistatic over the supernodulation shoot. This example of epistasis is explained in terms of the developmental pathway to nodule formation. The double recessive mutants of crosses between nts382 and the non-nodulation mutants were confirmed by grafting their shoots onto the wild-type roots. This resulted in supernodulation. The non-nodulating double mutant DM49 has some growth characteristics distinguishable from the parental nod49 which suggests that supernodulation itself may not be the sole reason for decreased growth in nts382.

Plants that are heterozygous for a non-nodulation locus display wild-type nodulation in the normal autoregulated background, thus indicating the recessive inheritance of non-nodulation. However, in the nts382 background, the non-nodulation mutations are incompletely dominant. A model is proposed to explain the possible existence of incomplete dominance of the non-nodulation mutants at the microscopic level and their recessive nature for nodulation in the autoregulated background. The limitations of selection for defective infection mutants in an autoregulated background are emphasised since only null mutants will be reliably detected. Selection of either bacterial or plant mutants in a supernodulating (not autoregulated) background has the advantage that leaky mutants in sub-epidermal cell divisions will be detected because a greater proportion of the sub-epidermal cell divisions advance to visible nodules.

Publications

Carroll, B.J., Mathews, A., Whitmore-Smith, D. and Gresshoff, P.M. (1986) Nodulation and nitrate metabolism mutants of soybean. Conference on Molecular and Cellular Biology of the soybean. p. 23.

Delves, A.C., Mathews, A., Day, D.A., Carroll, B.J. and Gresshoff, P.M. (1985) Root and shoot factors affecting nodulation in soybean mutants. Genetics Society of Australia, 32nd Annual Conference, Sydney, Australia. p. 75

Delves, A.C., Mathews, A., Day, D.A., Carter, A.S., Carroll, B.J. and Gresshoff, P.M. (1986) Regulation of the soybean-*Rhizobium* nodule symbiosis by shoot and root factors. *Plant Physiol.* 82, 588-590.

Mathews, A., Carroll, B.J., Bauer, W.D. and Gresshoff, P.M. (1986) rj_6 - A new gene conditioning non-nodulation in soybean. The Australian Institute of Agricultural Sciences Occasional Publ. No. 25 (additional sheet).

Mathews, A., Carroll, B.J. and Gresshoff, P.M. (1987) Characterization of the non-nodulation mutants of soybean *(Glycine max* (L.) Merr.*)*: *Bradyrhizobium* effects and the absence of root hair curling. *J. Plant Physiol.* 131, 349-361.

Mathews, A., Carroll, B.J. and Gresshoff, P.M. (1987) Host genetics of non-nodulation mutants of soybean. Genetics Society of Australia, 34th Annual Conference, Canberra, Australia, p.70.

Mathews, A., Carroll, B.J. and Gresshoff, P.M. (1987) Nodulation mutants of soybean. In: D.P.S. Verma and N. Brisson, eds., Molecular Genetics of Plant-Microbe Interactions. Martinus Nijhoff Publ., Dordrecht, The Hague, pp. 94-95.

Mathews, A., Carroll, B.J., McNeil, D.L. and Gresshoff, P.M. (1985) Isolation and characterization of non-nodulating mutants of soybeans. In: H.J. Evans, P.J. Bottomley and W.E. Newton, eds., Nitrogen Fixation Research Progress. Martinus Nijhoff publ., Dordrecht, The Hague, p. 45.

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CHAPTER 1

GENERAL INTRODUCTION

Symbiotic nitrogen fixation in legumes is a tightly coordinated interaction between leguminous plants and rhizobia*. Dinitrogen (N_2), a relatively inert gaseous form of nitrogen, which constitutes more than three quarters of the atmosphere, is converted by this process into a chemical form (ammonia) utilizable by plants. Legumes in conjunction with bacteria which belong to the family Rhizobiaceae such as *Rhizobium, Bradyrhizobium* and *Azorhizobium* (see Jordan, 1984) possess the ability to form specialized plant organs called nodules. In these nodules the physiological milieu needed for nitrogen fixation is established by a biochemical coordination of processes in both the plant and the bacterium. Symbiotic nitrogen fixation in legumes contributes a major portion of the biologically fixed nitrogen available for agriculture and can account for several hundred kilograms of nitrogen fixed per hectare per annum (Vincent, 1974; Beringer, 1984). The contribution of nitrogen fixation to crop production is either through the provision of nitrogen for the legume or through a residual effect on the subsequent crop (Dart, 1984).

More than a century ago, it was observed that pea plants grew better in nitrogen deficient soils if they had nodules on their root system (c.f. Aulie, 1970). The bacterium then called *Bacillus radicicola* but now known as *Rhizobium leguminosarum* was isolated from these nodules (see Dart, 1974). In 1915, Vorhees noted that "different varieties of the same legume bear different and definite powers of resistance to association with symbiotic bacteria". For decades attempts aimed at the improvement of nitrogen fixation have been concerned only with the genetic improvement of the microsymbiont. Recently, the contribution of the host genotype has received attention from agronomists and biologists. However, our knowledge of the host genetics of infection and nitrogen fixation is far from being complete and lags behind the advances made with the prokaryotic partner (Atkins, 1986).

Genetic factors influencing nodulation and nitrogen fixation are present in the legume genome and any improvement in either nodulation and/or nitrogen fixation would require an identification and manipulation of the legume genetic material (LaRue, 1980; Gresshoff and Delves, 1986).

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* The term rhizobia when used in this thesis refers collectively to the slow growing *Bradyrhizobium* species and the fast growing *Rhizobium* species. For reasons of familiarity and convenience, *R. trifolii* is used instead of the new nomenclature *R. leguminosarum* biovar *trifolii*.

1.1 Stages of nodule initiation and development in legumes

Nodule initiation in legumes begins with the initial encounter between the microsymbiont and the legume and proceeds through several steps involving bacterial adsorption to the root surface, the response of root hairs and infection of root hairs (Vincent, 1980; Pueppke, 1986). The entire process of nodule ontogeny can be divided into three major categories: preinfection, infection and nodule development. The modified phenotypic sequence of Vincent (1980) for the root nodule symbiosis used here is listed in Table 1.1.

1.1.1 Preinfection events

This category consists of (i) root colonization (Roc), (ii) root adhesion (Roa), (iii) *nod* gene induction (Ngi), (iv) sub-epidermal cell division (Scd), root hair branching (Hab) and root hair curling (Hac) (Table 1.1). In addition, chemotaxis (Cht) of motile strains (Bowra and Dilworth, 1981) has been included in the preinfection events.

1.1.1.1 Chemotaxis (Cht)

Chemotaxis is thought to be the first step in nodulation and nitrogen fixation, but is considered to be neither host specific nor mandatory for nodulation (Currier and Strobel, 1976). The non-specificity of chemotaxis has been confirmed by Gaworzewska and Carlile (1982). Chemotaxis is not restricted to homologous rhizobia - legume systems and hence is not an important determinant in host specificity. However, as most rhizobia possess flagella, motility has been considered as a possible factor in rhizosphere competition (Dowling and Broughton, 1986). The competitive advantage for motile strains

Table 1.1 Analysis of infection events in soybean.
Modified from Vincent (1980).

Table 1.1

Stage	Abridged description	Phenotypic code
I. Preinfection		
1. Chemotaxis and motility	Chemotaxis	Cht
2. Multiplication on root surface ('rhizoplane')	Root colonization	Roc
3. Attachment to root surface	Root adhesion	Roa
4. Induction of *nod* gene expression by flavones and/or isoflavones	*Nod* gene induction	Ngi
5.(a) Division and multiplication of the cortical cell	Sub-epidermal cell division	Scd
(b) Branching of root hairs	Hair branching	Hab
(c) 'Marked' curling of root hairs	Hair curling	Hac
II. Infection and Nodule formation		
6. Formation of infection thread	Infection	Inf
7. Host blockage of nodule formation	Autoregulation	Aut
8. 'Intracellular' release of rhizobia from infection thread	Bacterial release	Bar
9. 'Intracellular' multiplication of rhizobia and development of full bacteroid form	Bacteroid development	Bad
III. Nodule Function		
10. Reduction of N_2 to NH_4^+ (Nitrogenase)	Nitrogen fixation	Nif
11. Complementary biochemical and physiological functions	Complementary functions	Cof
12. Persistence of nodule function	Nodule persistence	Nop

above non-motile strains has been observed in co-infection experiments (Ames and Bergman, 1981). Likewise, Soby and Bergman (1983) observed that when a motile wild-type strain and its non-motile mutant were introduced into different sterile soils, chemotaxis and motility were required for a more efficient spreading of the rhizobia throughout the soil. Similarly, in sand-vermiculite mixture, a non-motile mutant of *B. japonicum* serogroup 110 formed only 20 per cent of the nodules when compared to the motile parent inoculated at equal cell titres (Hunter and Fahring, 1980). Therefore, chemotaxis and motility are not a prerequisite for nodulation but could be advantageous in competition in the soil.

1.1.1.2 Root colonization (Roc)

The establishment of a population of rhizobia in the seedling rhizosphere is one of the events leading to successful nodulation (Brockwell *et al.*, 1985). According to Bauer (1981), infection of soybean by *B. japonicum* probably begins with the response of the bacterium to nutrients and signal compounds from the root. Stimulation of the growth of the microsymbiont in the rhizosphere of the legumes has also been studied. A marked stimulation of rhizobia numbers in the rhizosphere of the legume compared to the soil more distant from the legume root has been reported (see Dart, 1977). This stimulation is most noticeable in poor soils and for homologous rhizobia (Hely and Brockwell, 1962).

Legume root exudates are implicated as stimulators of rhizobial growth and multiplication in the rhizosphere. Vitamins and amino acids released from the root have been found to be required by several strains of rhizobia . For example, thiamine and biotin are required by many strains of rhizobia for their growth (West and Wilson, 1939a,b; Graham, 1963) and homoserine released by pea roots (van Egaraat, 1975b) is utilized selectively by *R. leguminosarum* and is considered to stimulate its growth on the pea rhizoplane. van Egaraat (1975a) reported exudation of ninhydrin-positive compounds in peas from the tips of main roots, lateral roots and from the wounds caused by the emergence of lateral roots from the main root. In the case of nodulated plants the root exudates stimulate rhizobial growth in the rhizosphere and enable the establishment of a favourable environment for nodulation (Currier and Strobel, 1976).

1.1.1.3 Root attachment (Roa)

Some forms of binding or attachment of the microsymbiont to the legume root are thought to be important in the recognition between specific partners. Multiple mechanisms of bacterial attachment to the root hairs are known. Of these, the two major forms are firm and loose attachment. van Rensberg and Strijdom (1982) reported that firm attachment was host-specific in *R. meliloti* -alfalfa and *R. trifolii*- clover systems while loose attachment was non-specific. On the other hand, Vesper and Bauer (1985) observed that firm attachment of bradyrhizobia to the soybean roots is neither host-specific nor host-selective. The importance of loose attachment or association between the *Bradyrhizobium* and the root was implicated by Bauer *et al.* (1985) while studying the attachment of strain 1007, a derivative strain of USDA110 which lacks the firm attachment ability. Strain 1007 nodulates as efficiently as USDA110. This observation questions the need for any form of firm attachment in the infection process. The substances or structures that are involved in loose attachment of rhizobia to the root surface is not known (Bauer *et al.*, 1985).

The molecular mechanism of attachment and recognition has been studied by several groups (Halverson and Stacey, 1986). Lectins which are proteins produced in legume roots and seeds have been implicated in the specificity of attachment as they can bind to specific carbohydrate moieties of the rhizobia. Host lectins are reported to bind to cell surface polysaccharides, such as lipopolysaccharides (LPS), capsular polysaccharides (CPS) and extra-cellular polysaccharides (EPS), from homologous and heterologous rhizobia (Bohlool and Schmidt, 1974; Dazzo and Hubbell, 1982). The production of these receptors at different phases of rhizobial cell growth has been reported by Sherwood *et al.* (1984) and presumably occurs in the rhizosphere of the legume host. Dazzo *et al.* (1984) demonstrated a strong correlation between lectin binding and infectivity in the *R. trifolii* attachment to clover roots. They proposed a two step model for attachment comprising an initial non-specific adherence followed by a specific polar attachment involving lectins which they postulated to be essential for normal infection and nodulation. Soybean lectins have also been postulated to be important in the attachment of

B. japonicum cells to the host root surfaces (Stacey *et al.*, 1980) but they are not the sole agents involved in attachment (Stacey *et al.*, 1984).

The majority of the earlier studies conducted on recognition have used seed lectins rather than root lectins. The isolation of root lectins is useful in determining the involvement of lectins in recognition and specificity of the legume-rhizobia symbiosis. Root lectin from clover has been isolated and subsequently shown to interact specifically with a cell surface polysaccharide of *Rhizobium trifolii* (Dazzo and Brill, 1977; Dazzo *et al.*, 1976; Dazzo *et al.*, 1979). It has been shown that *Lotononis bainesii* root lectin binds to infective rhizobia (Law and Strijdom, 1984) while the seed lectin does not bind to the cell surface of infective *Rhizobium* sp. (Law and Strijdom, 1977). In soybean and sweet clover, root lectins which bind to their infective rhizobial strains, have been reported by Stacey *et al.*(1980) and Seegers and LaRue (1985). Halverson and Stacey (1985) observed a lectin mediated phenotypic reversal of a slow-to-nodulate strain of *B. japonicum* which nodulated further down on the root.

The lack of specificity in the attachment of bradyrhizobia to soybean roots has been confirmed by Vesper and Bauer (1985) who found that heterologous strains of bradyrhizobia incapable of infection or nodulation of soybean could attach to the soybean root segments and root hairs as readily as the homologous strains. Likewise, Pueppke (1984) observed that rhizobial strains attach comparatively well to soybean and cowpea roots irrespective of which of these legumes they nodulate. Several studies have reported that rhizobia attach comparatively well to the surfaces of host and non-host roots alike (Chen and Phillips, 1976; Shimschick and Hebert, 1978; Pueppke, 1984; Badenoch-Jones *et al.*, 1985; Mills and Bauer, 1985; Anolles and Favelukes, 1986).

The involvement of pili in the firm attachment of *B. japonicum* has been reported (Bauer *et al.*, 1985). Extracellular cellulose microfibrils have been implicated in tight binding of the rhizobia to the root surface (Higashi and Abe, 1980; Matthysse, 1983). Therefore, the nature and role of attachment in the establishment of an effective legume-rhizobia symbiosis has not been fully determined.

1.1.1.4 Root exudate signals and induction of nodulation genes in the microsymbiont (Ngi)

Bhagwat and Thomas (1982) and Halverson and Stacey (1984a) observed several rhizobial strains which exhibit a delay in nodulation. Pretreatment of these strains with the host root exudates has been shown to reverse the delayed nodulation phenotype (Bhagwat and Thomas, 1982; Halverson and Stacey, 1984b), indicating that the root excretes substances which condition the bacteria for nodulation. Bhagwat and Thomas (1982) observed that the preincubation of strain 32H1 in cowpea root exudates for 4 to 6 h prior to inoculation of cowpea seedlings decreased the time required for nodule formation. The mid-logarithmic phase cultures of strain 1001 initiated an immediate nodulation response on cowpea, while the stationary-phase cultures initated a delayed nodulation response. It was observed that when the stationary-phase culture of this strain was preincubated in cowpea root exudates for 4 to 16 h prior to inoculation, it could initiate nodulation similar to the mid-logarithmic phase cultures (Bhagwat and Thomas, 1983; 1984) suggesting that components of cowpea root exudates induce a structural change in the CPS of strain 1001 (Bhagwat and Thomas, 1982).

Bradyrhizobium japonicum strain HS111 is a slow-to-nodulate mutant (Stacey *et al.,* 1982) and is probably defective in nodule initiation and in the rate of nodule development (Halverson and Stacey, 1984a). The mutant phenotype was phenotypically reversed by preincubation in soybean root exudates and the active component was determined to be a galactose-specific soybean root lectin (SBL) (Halverson and Stacey, 1984a; 1984b; 1985; 1986). Therefore, lectins may function as a signal molecule which induces the expression of rhizobial genes required for nodulation. Molecular analyses indicated that components of legume exudates, such as flavones (Peters *et al.,* 1986; Redmond *et al.,* 1986) or flavanones (Firmin *et al.,* 1986) can induce *nod* genes in *R. meliloti, R. trifolii* and *R. leguminosarum.* Kosslak *et al.*(1987) identified the isoflavones daidzein and genistein as major components in soybean root extracts which are responsible for *nod* gene induction in *B. japonicum.* Mutations in these *nod* genes eliminate the ability of the microsymbiont to nodulate its legume host.

1.1.1.5 Root hair branching (Hab) and root hair curling (Hac)

Various degrees of deformation such as branching, lobing or curling of root hairs have been noticed after inoculation with rhizobia (Yao and Vincent, 1976). Yao and Vincent (1969) categorized root hair deformation into three classes based on the degree of deformation. These are: (a) branching - wherein root hairs have lateral branches and short outgrowths; (b) moderate curling - wherein root hairs are curled to less than 360^{o}; and (c) marked curling - where the root hairs are curled 360^{o} or more. Marked curling is reported to be the result of living cells of homologous rhizobia interacting with growing root hairs. The first two categories do not require living cells of rhizobia. In white clover and alfalfa, exposure of the roots to culture fluids of nodule inducing and sometimes non-nodule inducing rhizobia can induce such deformations (Sahlman and Fahraeus, 1963; Yao and Vincent, 1969).

The region of most active curling in the deformed root hair is the point where rhizobia gain entry into the hair. Several reports indicate that infections can originate wherever the bacteria get trapped between closely appressed walls and such an entrapment is generally achieved by the marked curling of root hairs (Napoli and Hubbell, 1975; Higashi and Abe, 1980; Callaham and Torrey, 1981; Robertson, 1981; Turgeon and Bauer, 1982; Ridge and Rolfe, 1986). The occurrence of infections in moderately curled root hairs have also been reported by Nutman (1959), Higashi and Abe (1980) and Dazzo and Truchet (1983).

The nature of factor(s) causing deformation of root hairs is not fully understood. Hubbell (1970) observed that cell free exo-polysaccharide (EPS) from *R. trifolii* cultures induced curling and deformation of *Trifolium fragiferum* root hairs and the extent of deformation was proportional to the concentration of the crude polysaccharide in the growth medium. Slight deformation of root hairs was induced by the EPS obtained from a non-infective strain of *R. trifolii*. Yao and Vincent (1976) reported the presence of heat labile and heat stable deforming factors in the culture fluids. They confirmed the observation of Ljunggren (1969) that, in addition to the high molecular weight substances, there are heat stable and dialyzable substances in the culture fluids of

R. trifolii that induce deformation in clover root hairs. The factor inducing root hair branching was separated from that causing moderate curling by Yao and Vincent (1976). Therefore, the deformation of root hairs is caused by more than one substance present in the culture filtrates. Bhuvaneswari (1984) characterized low molecular weight root hair branching factors present in the culture filtrates of *R. trifolii* cultured for 48 h in the presence of white clover seedlings and suggested that the root hair branching factor(s) may be oligosaccharides of various sizes.

'Shepherd's crook' formation is the tight curling (more than 360^{o}) response of the root hair tip and is induced by living rhizobia which are specifically capable of infecting the legume root (Yao and Vincent, 1976). Tight curling of the root tip requires either attachment or close proximity to the root surface of viable, homologous and infective rhizobia. Serial thin sections have revealed the enclosure of rhizobia in the central overlap of the 'knot' created by the 'shepherd's crook' (Napoli and Hubbell, 1975).

There is evidence to indicate that the initial cell division in nodule ontogeny is also a preinfection event and this stage is discussed in detail in Section 1.1.3.

1.1.2 Infection

As early as 1887, Ward observed that nodulation of *Vicia faba* roots by *Rhizobium* was initiated with the infection of the root hairs by the formation of a bacterial thread which grew into the cortex of the root. Later in 1932, McCoy pointed out that root hairs show strong deformation shortly after inoculation of the root with nodulating bacteria and that a tubular thread continues through the root hair wall containing the bacteria. Since then a considerable amount of research has been directed towards characterizing the infection process.

1.1.2.1 Root hair infection

Infection threads are initiated at or near the growing tip of a root hair (Turgeon and Bauer, 1982). Initiation of the infection thread generally occurs

adjacent to the rhizobia trapped in the crook of the curled root hair. Invagination of the cell wall of the root hair occurs forming a pocket where bacteria are trapped in a matrix of unknown composition and origin (Callaham and Torrey, 1981). Thin sections revealed that the matrix material was separated from the root hair plasma membrane by plant cell wall material called the infection thread wall. The infection thread continues to grow down the root hair and through the cortical cells. Bacteria when inside the infection thread wall are surrounded by a matrix suggested to resemble extracellular polysaccharides either derived from the bacteria (Callaham and Torrey, 1981) or of plant origin (Robertson and Lyttleton, 1982; Turgeon and Bauer, 1985).

The question of whether the root hair curling process is a prerequisite for the initiation of the infection thread and whether hair curling could be a consequence of a localized infection by the rhizobia remains unanswered. There are reports of the initiation of cortical cell divisions during the root hair curling stage in the legume- rhizobia symbiosis (Truchet *et al.*, 1984; Calvert *et al.*, 1984). Mutants of rhizobia are known to induce root hair curling without the apparent formation of infection threads (Downie *et al.*, 1984) implying that curling might precede infection. On the other hand, Callaham and Torrey (1981) observed that infection threads can be initiated without root hair curling in some cases.

1.1.2.2 Alternative infection mechanisms

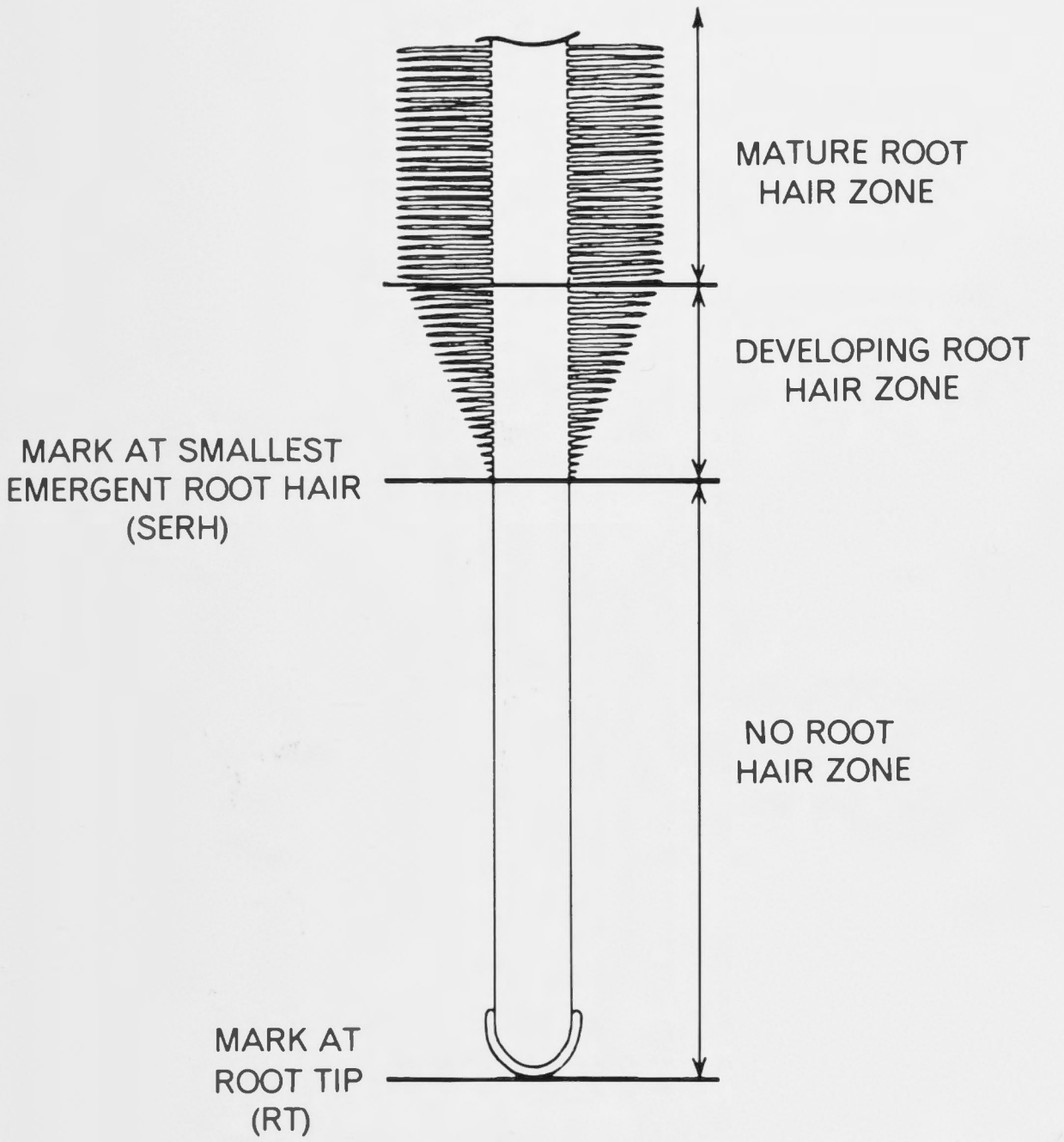
In 1940, Allen and Allen reported that nodules of peanut *(Arachis hypogaea)* occur at the junction of lateral roots. Several decades later, Chandler (1978) observed that rhizobia enter the root at the junction of the root hair and the epidermal cells. Here, the rhizobia divide rapidly within the cortical cells of the emergent lateral root and the invaded host cells divide repeatedly to form the nodule tissue (Chandler, 1978). In *Neptunia oleracea,* a water plant which lacks root hairs, nodules were formed on the adventitious roots and infection threads were initiated in the epidermal cells (Schaede, 1940). Similarly, occasional infection threads were observed in root epidermal cells of soybean (Bieberdorf, 1938) and clover (Nutman, 1959). In *Lupinus* sp., a mechanism of 'intercellular' infection has been suggested since infection threads have not been

observed (see Dart, 1974). *Aeschynomene indica* nodules formed on the stem base and on the roots arise near emerging lateral roots. Root hairs were absent and no infection threads were found in the nodules and the bacteria were spread by cell division (Arora, 1954). A similar mode of infection occurs in *Sesbania* stems where emergent adventitious roots are converted into nodules. In the non-legume *Parasponia* , nodulation by *Bradyrhizobium* occurs after the initiation of cell divisions in the root hair cells and in the epidermal and cortical cells close to the emergence of a 'dormant' lateral root primordium. Infections occur via infection threads but do not involve the root hair (Bender *et al.,* 1987).

1.1.2.3 Location of infectible cells in legumes

Infectibility of root hairs appears to be a transient phenomenon. In soybean, infection is confined to areas of the root which are actively growing as illustrated in Figure 1.1. In addition, infections in soybean occur more frequently in root hairs that had not formed at the time of inoculation (Bhuvaneswari *et al.,* 1980). The susceptibility to nodulation is lost within 4 to 6 h of inoculation in the entire region above the root tip mark. The susceptibility is also developmentally restricted and transient in several other legumes. The mature and developing root hair regions of soybean and cowpea are less susceptible to infection by rhizobia (Calvert *et al.,* 1984; Pueppke, 1983). Root hairs of soybean appear to have only one infectible period whereas in clovers there are two: one early and one late in hair development (Bhuvaneswari *et al.,* 1981). In white clover, *Rhizobium* infections resulting in nodules can also occur in root hair cells that are mature or developing (Bhuvaneswari *et al.,* 1981) and root hairs become susceptible to infection only after they develop lateral branches (Bhuvaneswari, 1984). Alfalfa is similar to white clover *(Medicago sativa)* in nodulation but it has a lower frequency of nodulation in the mature and developing regions (Bhuvaneswari *et al.,* 1981). In siratro *(Macroptilium atropurpureum)*, however, studies using growth pouches indicate a zone of infection approximately 2 cm on either side of the root tip mark. The fully elongated root hair zone at the time of inoculation does not develop nodules (Ridge and Rolfe, 1986). Likewise, in subterranean clover (*Trifolium subterraneum)*, the region immediately behind the root tip is the major infection target (Sargent *et al.,* 1987).

Figure 1.1 **Location of the most infectible cells in a soybean root** (Bhuvaneswari *et al.*, 1981). The most infectible region is the region between the root tip (RT) and the shortest emerging root hair (SERH). The mature root hair zone is not susceptible to infection.


MATURE ROOT
HAIR ZONE
DEVELOPING ROOT
HAIR ZONE
MARK AT SMALLEST
EMERGENT ROOT HAIR
(SERH)
NO ROOT
HAIR ZONE
MARK AT
ROOT TIP
(RT)

The culture age of bradyrhizobia plays a role in affecting the efficiency of infection in *Bradyrhizobium japonicum* strains USDA110ARS, USDA123 and USDA138 (Bhuvaneswari *et al.*, 1983). They observed that upon entering stationary growth phase these strains rapidily lost their ability to cause nodulation above the root tip mark indicating that the ability of these strains to initiate infection is delayed at this stage of growth. On the other hand, the culture age of strain 83 and 61A76 had relatively little effect on infectivity. Changes in infectivity with culture age were well correlated with changes in the proportion of cells in a culture capable of binding soybean lectin (Bhuvaneswari *et al.*, 1983).

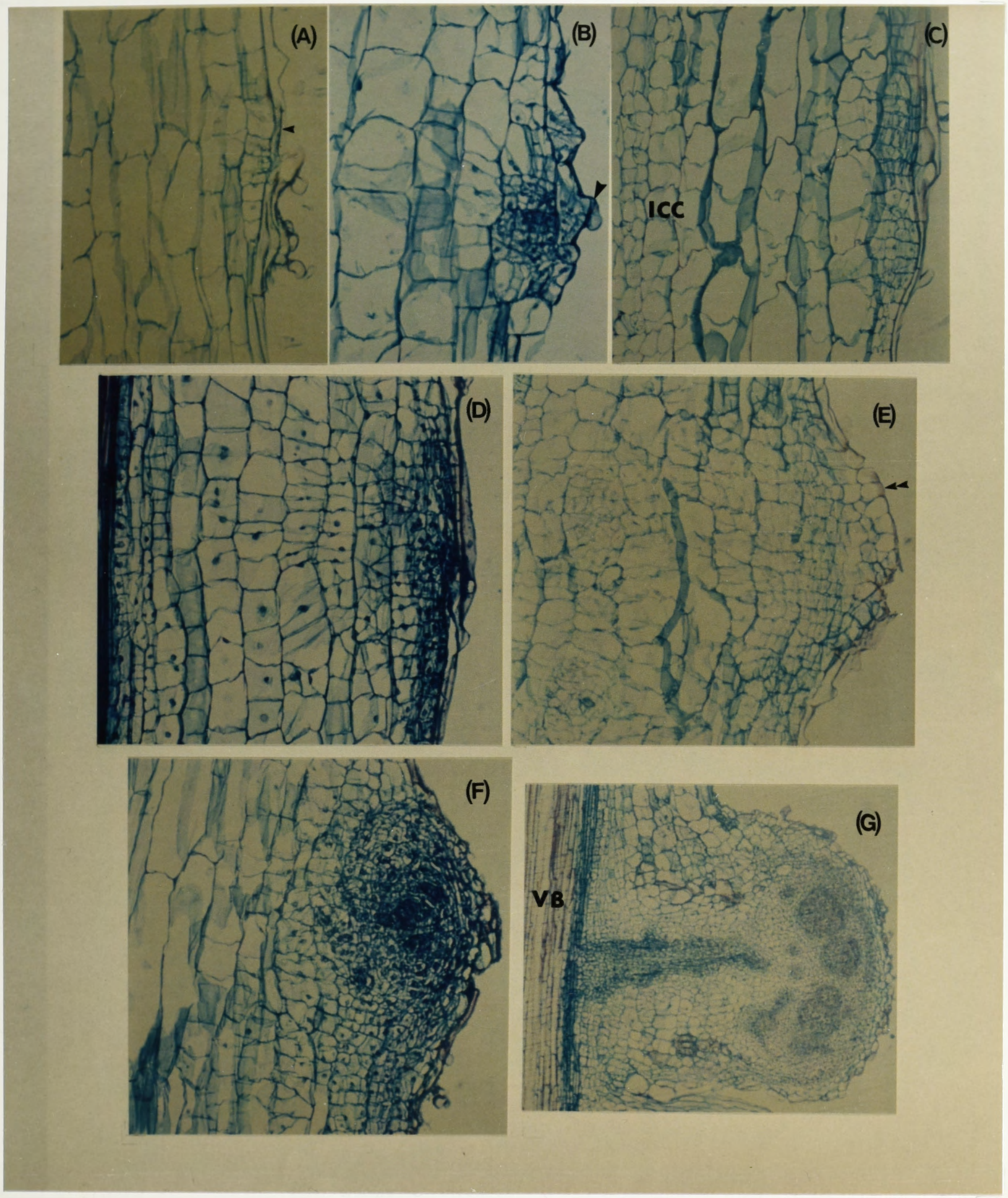
1.1.3 Initiation of the root nodule meristem

In most leguminous species, root nodules arise from cortical tissue (Bond, 1948) but in soybean the actual meristem is initiated in the non-mature root (Calvert *et al.*, 1984). A number of early studies focussed on early stages in the proliferation of root tissue in the formation of pea nodules (Wipf and Cooper, 1940; Torrey and Barrios, 1969). Libbenga and Harkes (1973) observed cell division in the inner cortex of pea at some distance from the advancing infection thread. Subsequently the infection thread has been observed to penetrate almost the entire depth of the cortex with the branches penetrating the meristematic area initiated earlier in the inner cortex. The cells are invaded by the bacteria released from the infection thread. New meristematic activity is initiated in adjoining cortical cells when the initial meristematic centre in the inner cortex is used for bacteroid formation. As development proceeds, more cortical layers contribute to the nodule with the peripheral layer, namely, the nodule cortex and the apical meristem of the nodule not invaded by bacteria. Nodule initiation in soybean is slightly different from that described so extensively for temperate legumes such as pea and white clover. Figure 1.2 shows the sequence of sub-epidermal cell divisions in soybean. It should be noted that the term 'sub-epidermal cell divisions' (scd) used in this thesis refers to the cell divisions in both the hypodermis and the cortex of the root. In soybean, the nodules are induced from sub-epidermal cell divisions close to the epidermis and meristematic activity terminates relatively early. The nodule growth is dependent on subsequent cell extension.

Figure 1.2 **Sequence of sub-epidermal cell divisions in soybean cv. Bragg.** Observations were taken 5 days after inoculation and culture in plastic growth pouches. Root segments were serially sectioned longitudinally. For further details see Chapter 5. The single arrow indicates the infection threads and the double arrow indicates a curled root hair.

(A) is stage I, (B) is stage II, (C) is stage III, (D) is stage IV, (E) is stage VI, (F) is stage VII and (G) is stage VIII in the sequence of sub-epidermal cell divisions and nodule formation as described by Calvert *et al.* (1984).

ICC - inner cortical cells
VB - vascular bundle


(A)
(B)
(C)
ICC
(D)
(E)
(F)
(G)
VB

Soybean, like other legumes, restricts the number of successful infections. Most actual infection sites (i.e. with infection threads) stop developing at stages prior to defined meristematic formation. A high proportion of infection events are pseudoinfections which are localized areas of sub-epidermal cell divisions not associated with an infection thread. They are initiated very frequently in the developing root hair zone, sparsely throughout young regions of the root and very rarely, if at all, in the mature root hair zone (Calvert *et al.*, 1984).

The first hypodermal cell division in soybean is completed 12 h after inoculation (Calvert *et al.*, 1984) and root hair curling is seen 6 h after inoculation (Turgeon and Bauer, 1982; 1985). Hence, it had been suggested that the mechanisms which initiate penetration and infection thread formation may be quite different from those that induce cortical cell divisions (Calvert *et al.*, 1984). The infection thread formation was observed 18 h after inoculation in soybean. Host cell division could thus be initiated by the bradyrhizobia soon after inoculation and long before infection thread formation. The existence of sub-epidermal cell divisions without associated root hairs suggests that the induction of at least the first cortical cell division may be independent of root hair emergence and induced root hair deformation. Truchet *et al.* (1984) and Hirsch *et al.* (1984) came to the same conclusion from anatomical analyses of pseudonodules on alfalfa induced by either *Agrobacterium tumefaciens* or *Escherichia coli* containing nodulation genes from the *R. meliloti* symbiotic plasmid. Transconjugates were able to stimulate nodule meristem formation without necessarily causing root hair infection thread formation and in some cases root hair deformation. Bauer *et al.*(1985) suggested that the induction of sub-epidermal cell division precedes marked hair curling and infection since sub-epidermal cell divisions still occurred after a nucleopore membrane was interposed between the *Bradyrhizobium* and the roots permitting substances to move freely between the two. They concluded that homologous bacteria can initiate sub-epidermal cell divisions in the root even when they are physically separated from the root surface. This suggests that some diffusible substance excreted from the bradyrhizobia can induce cell divisions in the cortex of the root. No evidence was available to indicate that heterologous species can induce these divisions. Cytokinins have been implicated as inducers of sub-epidermal

cell divisions. In fact, Bauer *et al.* (1985) have shown that exogenous benzyladenine can induce sub-epidermal cell divisions in soybean.

1.1.4 Nodule formation

After cortical cells are stimulated to divide and have undergone some division, the nodule meristem is formed. In soybeans, rhizobia enter the newly divided host cells through the growth and branching of the infection thread (Goodchild and Bergersen, 1966; Basset *et al.,* 1977; Newcomb, 1981; Turgeon and Bauer, 1982; 1985). Bacteria are then released into the cytoplasm of cortical cells and are enveloped by a host derived peribacteroid membrane. The bradyrhizobia then differentiate into bacteroids. The multiplication of both the bacterial and host cells results in the formation of nodules. The mature bacteroids reduce atmospheric nitrogen into ammonia which is assimilated by the legume for growth and development. Along with bacteroid differentiation, nodule-specific proteins called nodulins are synthesized by the host during nodule formation. Of these, ENOD2 is induced at an early stage in the infection process while the others such as glutamine synthetase (GS), leghemoglobins, uricase (N35) and sucrose synthetase (N100) are induced later with the onset of nitrogen fixation (see Gresshoff and Delves, 1986; Halverson and Stacey,1986).

1.1.5 Autoregulation

External and internal factors control the number of nodules on the root system. Nodule initiation and the number, size and function of nodules are known to be inhibited by exogenously fixed forms of nitrogen such as nitrate (Thornton, 1936; Munns, 1968a,b,c; Vigue *et al.,* 1977; Wong, 1980; Streeter, 1981; Ralston and Imsande, 1983). Under optimal conditions for nodulation there are also internal control mechanisms operating in the control of nodulation. It has been observed that nodulation frequency

decreases to 20 per cent of the maximum in the regions of the root that are 10 to 15 h younger than the most susceptible zone of the root at the time of inoculation (Pierce and Bauer, 1983). It appears that the host plant has a mechanism of blocking infections in the root. A second inoculation of *Bradyrhizobium japonicum* 15 h after the initial inoculation does not increase the frequency of nodulation in the younger region of the root (Pierce and Bauer, 1983). Therefore, it appears that the host has a rapid regulatory mechanism which inhibits subsequent nodulation of younger root tissue. This is apparently caused by arresting the infection process (Calvert *et al.*, 1984). It is also known that the excision of mature nodules can stimulate the development of additional nodules (Nutman, 1952). However, most infections are arrested before they can develop into a nodule (Fahraeus, 1957; Nutman, 1962; Munns, 1968b; Dart, 1974), thereby limiting the number of nodules on a plant. This indicates that a mechanism is operative which regulates the number of nodules formed on the plant. This mechanism is called autoregulation (Carroll *et al.*, 1985 a,b).

Kosslak and Bohlool (1984) used a split root system to demonstrate systemic inhibition of nodulation on soybean. Inoculation of one side of a split root prior to inoculation of the other side resulted in the inhibition of nodulation on the latter, suggesting that autoregulation is a systemic response. However, the host signal molecule(s) involved in autoregulation have not been determined.

1.2 Root and shoot factors controlling nodulation

Several studies conducted have implicated shoot-root interactions in the control of nodulation (Kosslak and Bohlool, 1984; Lawn and Brun, 1974; Lawn and Bushby, 1982). Grafting techniques have been effectively used to study the effect of the shoot (scion) and root (rootstock) on nodulation. Clark (1957) showed that non-nodulation in the naturally-occurring non-nodulation mutant rj_1 of soybean is controlled by the root. This result was confirmed by Tanner and Anderson (1963) who also demonstrated that rj_1 neither lacks the cotyledonary factor necessary for nodulation nor contains a cotyledonary factor which inhibits

nodulation. Similarly, ineffective nodulation of Rj_2 (cv. Hardee) is determined by the root (Caldwell *et al.*, 1966). Degenhardt *et al.*(1976) observed that non-nodulation in the pea cv. Afghanistan was determined by the genotype of the host root.

However, Jacobsen *et al.* (1985) observed that the pea mutant K_{24} which is resistant to nodulation has a translocatable factor which inhibits nodulation. Likewise, Hely *et al.* (1953) reported that grafting of *Trifolium repens* (which nodulates well) onto *Trifolium ambiguum* (which nodulates poorly) resulted in the production of healthy nodules on the *Trifolium ambiguum* rootstock. These studies imply a possible role for the shoot in the regulation of nodulation. Plant growth regulators or other compounds synthesised in the root and/or the shoot are plausible candidates for regulating nodule formation.

1.3 Role of hormones in root nodule formation

The exact role of growth hormones in the nodulation process is not known. The observation that the inner cortical cells are stimulated to divide ahead of the advancing infection thread (Callaham and Torrey, 1981) raises the question of whether a diffusible division stimulus produced within the infected cell may be present. Furthermore, it has been demonstrated that sub-epidermal cell divisions in soybean do not require contact of the *Bradyrhizobium japonicum* cells with the root (Bauer *et al.*, 1985).

Auxins have been implicated as signals for cell division during root nodule initiation based on the following findings: (i) the ability of rhizobia species to convert tryptophan into indole 3-acetic acid (IAA) (Allen and Allen, 1958; Hartman and Glombitza, 1967; Dullaart, 1970); (ii) the high content of IAA in root nodules (Allen and Allen, 1958; Pate, 1958; Dullaart, 1970); and (iii) the release of IAA by rhizobia at the surface of roots of inoculated plants (Kefford *et al.*, 1960). Rhizobia also produce cytokinins (Phillips and Torrey, 1970) and cytokinins have been implicated to mimic bacteria in the induction of

sub-epidermal cell divisions (Bauer *et al.*, 1985). Gibberellin-like substances are also produced by cultures of *R. leguminosarum, R. trifolii* and *R. meliloti* (Katznelson and Cole, 1965). A high content of gibberellic acid (GA) in root nodules of *Pisum sativum , Phaseolus vulgaris* and *Lupinus luteus* has also been observed (Radley, 1961; Dullaart and Duba, 1970). Gibberellic acid (GA) excretion by nodules and root tips of *Phaseolus vulgaris* has been demonstrated (Radley, 1961). Williams and Sicardi de Mallorca (1982) observed high levels of GA-like substances in root nodules of soybean. Cis-trans abscisic acid (ABA) was also identified in the root and nodule extracts, but not in the extracts of bacterial cultures. Exogenously applied GA and the growth retardant ß-chloroethyltrimethylammonium chloride (CCC) are known to decrease nodulation and may be indicative of internal host mechanisms for controlling nodulation (Williams and Sicardi de Mallorca, 1984). Therefore, phytohormones could be involved in nodule initiation and also in autoregulation, although it is not certain whether their role is direct.

1.4 Host genetics

Several mutations affecting nodulation and nitrogen fixation are known in legumes. These are described in Table 1.2. In red clover *(Trifolium pratense* L.*)*, the gene *r* conditions resistance to nodulation and interacts with a cytoplasmic factor o (Nutman, 1949). Four genes affecting ineffective nodulation in red clover are reported (Nutman, 1954; 1957; 1968). Smith and Knight (1984) reported a gene conditioning a strain specific ineffective nodulation in crimson clover *(T. incarnatum* L.*)*. In pea *(Pisum sativum* L.*)*, resistance to nodulation and other symbiotic alterations are conditioned by at least eleven genes, of which *Sym*-1 is a dominant gene (Lie, 1971). Genes *sym*-3 and *sym*-6 condition ineffective nodulation in peas (Holl, 1975; Lie and Timmermans, 1983). Three genes affecting the number of nodules in pea have been reported (Gelin and Blixt, 1964; Jacobsen and Feenstra, 1984). Most of the genes affecting nodulation in pea are inherited as monogenic recessives. Resistance to nodulation in peas can be either strain or temperature dependent. The genes conditioning non-nodulation in chickpea *(Cicer arietinum* L.*)* are rn_1,

Table 1.2 **Plant genes affecting symbiotic nitrogen fixation in legumes.** These genes have been identified either as naturally-occurring variants (geographical accessions or spontaneous mutants of more recent origins) or in mutants induced by mutagenesis.

Table 1.2

Gene symbol	Origin	Nature of inheritance	Phenotype/Comments	Reference
			Red clover (*Trifolium pratense* L.)	
r	naturally-occurring	monogenic recessive	Resistance to nodulation. Interacts with a cytoplasmic factor σ. Homozygous *rr* is lethal, except in the presence of σ.	Nutman, 1949
i_1	"	"	Ineffective nodulation response with *R. trifolii* strain A, normal with strain B. Prevents bacteroid formation. Effectiveness can be restored to the $i_1 i_1$ by a recessive suppressor (m_1)	Nutman, 1954; Bergersen and Nutman, 1957.
ie	"	"	Ineffective response with several strains; conditions abnormal host cell divisions with no bacteroids, resembling tumours.	Nutman, 1957; Bergersen and Nutman, 1954
n	"	"	Ineffective response to some effective *R. trifolii* strains.	Nutman, 1968
d	"	"	Ineffective response to effective *R. trifolii* strains, producing smaller nodules, similar to *n*.	Nutman, 1968
			Crimson clover (*Trifolium incarnatum* L.)	
rt_1	naturally-occurring	"	Non-strain specific ineffective nodulation.	Smith and Knight, 1984
			Pea (*Pisum sativum* L.)	
Sym-1	naturally-occurring	monogenic dominant	Conditions a strain dependent resistance to nodulation below 20°C in cv.Iran.	Lie, 1971

Gene symbol	Origin	Nature of inheritance	Phenotype/Comments	Reference
			Pea (*Pisum sativum* L.)(cont)	
sym-2	Naturally-occurring	monogenic recessive	Conditions resistance to nodulation in cv. Afghanistan by *R. leguminosarum* strains from temperate soils, infected by some Middle East strains typified by strain TOM.	Lie, 1971; Holl, 1975; Kneen and LaRue, 1984a; Young *et al.*, 1982
sym-3	"	"	Conditions ineffective nodulation. Identified as an F_2 segregant from a cross between the cv. Afghanistan x Trapper.	Holl, 1975
sym-4	"	"	Conditions resistance to a single strain of *R. leguminosarum* in cv. Iran and Afghanistan.	Lie, 1984
sym-5	induced	"	Conditions non-nodulation by many *R. leguminosarum* strains tested. Temperature sensitive and nodulates if roots are maintained at 9-12°C. Forms few to no nodules when grown at 20/15°C day/night temperatures.	Kneen and LaRue, 1984b
sym-6	"	"	Conditions partly ineffective nodulation with *R. leguminosarum* strain F_{13} in cv. Afghanistan.	Lie and Timmermans, 1983
sym-7	induced	"	Conditions non-nodulation with all strains tested and at low temperatures.	Kneen *et al.*, 1986
sym-8	"	"	"	"
sym-9	"	"	"	"
sym-10	"	"	"	"
sym-11	"	"	"	"

Gene symbol	Origin	Nature of inheritance	Phenotype/Comments	Reference
			Pea (*Pisum sativum* L.)(cont)	
sym-12	induced	monogenic recessive	Conditions non-nodulation with all strains tested and at low temperatures.	Kneen *et al.*, 1986
sym-13	"	"	"	"
nod_1	naturally-occurring	"	Affects the number of nodules in cv. Parvus.	Gelin and Blixt, 1964
nod_2	"	"	"	"
nod_3	induced	"	Conditions an abnormally large number of nodules even in the presence of 15 mM KNO_3.	Jacobsen and Feenstra, 1984
			Chickpea (*Cicer arietinum* L.)	
m_1	induced	"	Conditions resistance to nodulation with all strains and at all temperatures tested.	Davis *et al.*, 1985; Davis *et al.*, 1986
m_2	"	"	Non-nodulation gene, conditions effective nodulation at 24°C and suppresses nodulation at 29°C.	"
m_3	"	"	Non-nodulation gene, conditions effective nodulation at 24°C and no nodulation at 29°C.	"
m_4	"	"	Conditions ineffective nodulation, resulting in the absence of specific acetylene reduction reduction activity at 24°C root temperature. The nodules lack visible leghemoglobin pigmentation.	Davis, 1985
m_5	"	"	Conditions an ineffective nodulation response with a severe reduction of nodule specific acetylene reduction at 24°C root temperature.	Davis, 1985

Gene symbol	Origin	Nature of inheritance	Phenotype/Comments	Reference
			Peanut (*Arachis hypogaea* L.)	
*	naturally-occurring	not monogenic recessive	Non-nodulating gene obtained while screening F_2 progeny of a cross between two nodulating cultivars.	Gorbet and Burton, 1979
*	"	duplicate recessive	Non-nodulation gene in peanut lines lacking root hairs.	Nigam *et al.*, 1980
			Alfalfa(*Medicago sativa* L.)	
nn_1nn_2	induced	two tetrasomically inherited genes	Conditions non-nodulation. Homozygous recessive alleles at all loci are necessary for non-nodulation.	Peterson and Barnes, 1981
in_1	"	single tetrasomically inherited genes.	This clone with ineffective nodules was obtained in a breeding program.	Peterson and Barnes, 1981
in_2	"	"	"	"
in_3	"	"	"	"
in_4 in_5	"	two recessive genes	This clone conditions ineffective nodulationwith the nulliplex condition at both loci required for ineffective nodulation	"
			Soybean (*Glycine max* (L.) Merr.)	
rj_1	naturally-occurring	monogenic recessive	Conditions non-nodulation with all strains of *Rhizobium* tested. Inoculation with a high cell number of *Rhizobium* results in a few nodules.	Williams and Lynch, 1954; La Favre and Eaglesham, 1984.
Rj_2	"	monogenic dominant	Conditions ineffective nodulation with *Rhizobium* strain c1 and 122 serogroups resulting in small white nodules. Ineffective nodulation is root controlled.	Caldwell, 1966; Caldwell *et al.*, 1966

Gene symbol	Origin	Nature of inheritance	Phenotype/Comments	Reference
			Soybean (*Glycine max* (L.)Merr.) (cont)	
Rj3	naturally-occurring	monogenic dominant	Conditions ineffective response to *Rhizobium* strain 33 resulting in small white nodules and nodule-like structures.	Vest, 1970
Rj4	"	"	Conditions ineffective nodulation response to *Rhizobium* strain 61.	Vest and Caldwell, 1972
*	"	"	Conditions ineffective nodulation of soybean cv. Kent with the fast growing strain *R. fredii* USDA205.	Devine, 1984

* not named

rn_2 and rn_3 while rn_4 and rn_5 condition ineffective nodulation (Davis, 1985; Davis *et al.,* 1985; Davis *et al.,* 1986). The genes affecting nodulation were identified after induced mutagenesis and are inherited as monogenic recessives. In peanut *(Arachis hypogaea* L.*),* two genes conditioning non-nodulation have been detected (Gorbet and Burton, 1979; Nigam *et al.,* 1980). For alfalfa *(Medicago sativa* L.) it has been shown that two tetrasomically inherited genes nn_1 and nn_2 condition resistance to nodulation while ineffective nodulation in alfalfa is determined by three genes (Peterson and Barnes, 1981).

In soybean *(Glycine max* (L.) Merr.*),* a single gene rj_1 conditioning non-nodulation and three genes conditioning ineffective nodulation have been reported (Williams and Lynch, 1954; Caldwell, 1966; Vest, 1970; Vest and Caldwell, 1972). The naturally-occurring non-nodulation mutation rj_1 was isolated by Williams and Lynch (1954) and was found to be inherited as a monogenic recessive. The initial characterization of this mutant was done by several researchers. It was observed that some strains of *B. japonicum* have a limited ability to nodulate this mutant (Clark, 1957). A correlation between the ability of *B. japonicum* strains to produce rhizobitoxine and nodulate has been reported (Devine and Weber, 1977; Devine *et al.,* 1983a). However, the results of La Favre and Eaglesham (1984) disproved this correlation. They also observed that inoculation with a high cell number of *B. japonicum* increased nodulation on this mutant. Clark (1957) and Tanner and Anderson (1963) reported that the non-nodulation trait was root controlled. The excretion of an inhibitory compound by the roots of the rj_1 mutant which decreased nodulation of the near isogenic line (Rj_1) was reported by Elkan (1961). However, Eskew and Schrader (1977) were unable to repeat this observation and this outcome will be discussed later in Chapter 5. The genetic linkage between the rj_1 locus controlling non-nodulation and the *F* locus determining fasciated stem in soybean has been established and found to be separated by a distance of 40 ± 2.2 genetic map units on linkage group 11 (Devine *et al.,* 1983b). Several attempts have been made in the past to characterize the nodulation resistant mutant rj_1 but the nature of the blockage in the nodulation process in this mutant has not been determined.

Three stable non-nodulation mutants of soybean were isolated by Carroll *et al.* (1986) following ethyl methanesulphonate (EMS) mutagenesis of the seeds of the soybean cv. Bragg. Of these mutants, nod49 was obtained from a mixture of M_2 populations screened in the field. Mutants nod772 and nod139 were isolated in the greenhouse from segregating EMS-derived M_2 families. The supernodulation and nitrate tolerant symbiosis mutant nts382 was also isolated in the same mutagenesis program. Mutant nts382 is a mutant in autoregulation and behaves as a single recessive gene (Carroll *et al.*, 1985a,b). The precise stage of alteration in nodulation and the genetics of these mutants was not known.

1.5 Objectives of the investigation

The main objective of this study was to investigate the host contribution to the initiation of nodules in the soybean-*Bradyrhizobium japonicum* symbiosis using three induced non-nodulation mutants of soybean (nod49, nod139 and nod772), a supernodulation and nitrate tolerant symbiosis mutant (nts382) and the naturally-occurring non-nodulation mutant rj_1. The characterization of these mutants was done giving emphasis to the preinfection and infection events in order to broaden the understanding of nodule initiation in soybean. The site of alteration in nodulation in these mutants was studied as well as, the inheritance and the genetic interaction of the supernodulation and the non-nodulation mutants.

The thesis is divided into the following chapters and arranged in such a manner that each experimental chapter (Chapters 3-7) represents an anticipated publication.

Chapter 2 contains the general materials and methods used in this investigation. Specialized materials and methods are described in individual results chapters.

Chapter 3 describes experiments which indicate that the mutations in the soybean mutants are nodulation specific. In addition, the inheritance of these mutants is demonstrated and whether or not they are affected in the same gene as the naturally-occurring non-nodulation *rj*$_1$ mutation described by Williams and Lynch (1954) is determined.

Chapter 4 presents the general characterization of the soybean mutants and attempts that were made to suppress non-nodulation in the non-nodulation mutants.

Chapter 5 deals with the characterization of the early infection events in nodulation in the soybean mutants as compared to the wild type and the location of the developmental site of alteration of the nodulation process.

Chapter 6 reports on grafting experiments done to determine tissue (root vs. shoot) control of the nodulation mutants since phytohormones and other compounds that could affect nodulation are synthesized in both the shoot and the root.

Chapter 7 presents the genetic interaction of the supernodulation mutant nts382 and the non-nodulation mutants. The identification of double recessive mutants and the demonstration of epistasis and incomplete dominance of the non-nodulation mutants nod49, nod139 and nod772 in a supernodulation background are detailed.

Chapter 8 contains the general discussion of this study including its relevance to nodulation in the wild type and some thoughts on future prospectives.

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter describes the plant material and general materials and methods used in these studies. Details of specialized techniques and data analyses are described in the relevant chapters.

2.1 Plant material

Soybean (*Glycine max* (L.) Merr.) cultivar Bragg, its ethyl methanesulphonate induced non-nodulating mutants nod49, nod139 and nod772 (Carroll *et al.,* 1986), the supernodulating and nitrate tolerant symbiosis mutants nts382, nts1116 and nts1007 (Carroll *et al.,* 1985 a,b) have been used for the experiments in this investigation. The naturally-occurring rj_1 mutation (Williams and Lynch, 1954) in the genetic background of the cultivar Lee was used for comparison purposes. Seeds of rj_1 (Lee) and Clark were obtained from Ian Rose of New South Wales Department of Agriculture, Narrabri, Australia. The soybean cultivar Williams was used in the grafting studies along with an intermediate supernodulator nts1116 and a supernodulating mutant nts1007 (Carroll *et al.,* 1985b).

2.2 Plant culture

Plants were cultured in the glasshouse using a 2 : 1 mixture of sand and vermiculite as a growth medium. Herridge's plant nutrient solution (Herridge, 1977) was used in these studies. The composition of the full strength Herridge's nutrient solution is contained in Table 2.1. A half strength solution of all the nutrients was applied to the seedlings for the first two weeks of plant growth, with the exception of $CaCl_2$ which was administered at full strength. During the subsequent weeks, the plants received a full strength of all the nutrients.

Nodulation tests were conducted in Leonard jars. The Leonard jar assembly consisted of a conical top compartment containing vermiculite which dipped into

Table 2.1 **The composition of Herridge's plant nutrient solution (Herridge, 1977).** The pH was adjusted to 7.0.

Table 2.1

Chemical[a]	Final Concentration (mg. l^{-1})
KH_2PO_4[b]	17.0
K_2HPO_4	21.8
KCl[b]	18.7
$MgSO_4.7H_2O$[b]	122.3
$CaCl_2$	27.7
Ferric monosodium salt of EDTA[c]	8.7
H_3BO_3[d]	71.5×10^{-2}
$MnCl_2.4H_2O$[d]	45.3×10^{-2}
$ZnCl_2$[d]	2.8×10^{-2}
$CuCl_2.2H_2O$[d]	1.3×10^{-2}
$NaMoO_4.2H_2O$[d]	0.6×10^{-2}

[a] chemicals were prepared as stock solutions and diluted in tap water

[b] administered from 1 M stock solutions

[c] administered from a 4000 times stock solution

[d] these chemicals were collectively prepared in a 4000 times stock solution

a reservoir containing half strength Herridge's nutrient solution. A cotton wick was inserted through the vermiculite into the reservoir. The neck of the top compartment was closed with a cotton plug. The entire assembly was wrapped in paper and autoclaved prior to culture (Vincent, 1970). Plants were also tested for nodulation in magenta jars which are similar to the above assembly. The main disadvantage of the magenta jars is the smaller reservoir volume. Plants used in pouch experiments were watered with Jensen's nutrient solution (Jensen, 1942). The composition of this plant nutrient solution is listed in Table 2.2.

2.3 *Bradyrhizobium* and *Rhizobium* strains

Bradyrhizobium japonicum strain USDA110 was used for most of the experiments in this study. This strain was supplied by Dr. J. Streeter (Ohio, U.S.A.). Strain USDA1-110ARS was used in the attachment studies and was obtained from Dr. W.D. Bauer (Ohio State University, Ohio, USA). Rj 138-1 (=USDA138) was obtained from Dr. E. Appelbaum (Agrigenetics, Madison, U.S.A.). Other *Bradyrhizobium* and *Rhizobium* strains used in this study were obtained from Mr. John Brockwell (CSIRO, Canberra). These were CC123 (=USDA123), CC127 (=USDA127), CC707 (=USDA7), CC708 (=Nitragin 61A76), CC709 (=Nitragin 61A77), CC722 (=USDA122), CC726 (=USDA31), CC724 (=USDA4), CC729 (=USDA76), CC731 (=USDA117), CC736 (=USDA192), CC737 (=USDA193), CC739 (=USDA194), CC740 (=USDA205), CC741 (=USDAWB1), CB1795 (=USDA46), CB1809 (=USDA136), CC1601d (ex. *Glycine clandestina*), CC1602d (ex. *G. tabacina*), CC1603d (ex. *G. tomentella*), CC1601b (ex. *G. canescens*) and cowpea strains NGR234, 32H1 and CB756. *Parasponia Bradyrhizobium* strain ANU289 was also used in the nodulation experiments in Chapter 4.

2.4 Rhizobia culture

Stock cultures were maintained on yeast extract mannitol (YEMA) agar (Vincent, 1970) slopes at 4^{o}C. The cultures were checked regularly and subcultured every six months. Colonies were also checked by streaking them on

Table 2.2 **The composition of Jensen's plant nutrient solution (Jensen, 1942).** The pH of the solution was adjusted to 6.8.

Table 2.2

Chemical	Concentration g. l^{-1}
$CaHPO_4$	1.0
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$FeCl_3$	0.1
Gibson's trace elements	1 ml*

***Gibson's trace element solution (Gibson, 1963)**

H_3BO_3	2.86 g. l^{-1}
$MnSO_4$	2.03 g. l^{-1}
$ZnSO_4.7H_2O$	220 mg. l^{-1}
$CuSO_4.5H_2O$	80 mg. l^{-1}
$H_2MoO_4.H_2O$	90 mg. l^{-1}

YEMA plates containing Congo red (Vincent, 1970) and Luria broth with glucose (LBG)(Miller, 1972) plates to study their colony characters and their inability to grow on LBG. The composition of YEMA containing Congo red and Luria broth with glucose are contained in Tables 2.3(i) and 2.4, respectively.

2.5 Rhizobial inoculation of plants

An inoculation loop full of culture was picked up from the YEMA slope and streaked on fresh YEMA plates which were incubated at 28^{o}C. Single colonies were harvested and grown in YEM broth at 28^{o}C on a rotary shaker adjusted to 150 rpm. Mid-logarithmic phase cultures were used in the experiments. Cultures were plated on YEMA plates after serial dilution in sterile distilled water to obtain the required cell numbers. This method of obtaining the required cell numbers of rhizobia was used for both the Leonard jar and pouch experiments. For the attachment studies, *B. japonicum* strain USDA1-110ARS was grown in yeast extract mannitol gluconate (YEMG) broth to early logarithmic phase and plated onto YEMGA plates containing 500µg. ml^{-1} streptomycin. The composition of YEMG is contained in Table 2.3 (ii) which is a modification of YEMA of Vincent (1970) by Bhuvaneswari *et al.* (1980).

Bradyrhizobium japonicum strain USDA110, grown in Bergersen minimal medium (BMM) liquid culture, was used to prepare the peat inoculant. The composition of BMM is detailed in Table 2.5. Sterile dry peat bags weighing 40 g were obtained from Agricultural Laboratories Pty. Ltd., Regents Park, New South Wales. The liquid culture of USDA110 was used either diluted or undiluted to inject the peat bags at the rate of 45 ml per bag. The inoculated bags were incubated in the dark at 28^{o}C for 10 days. Generally, each peat bag contained 10^8 to 10^9 viable cells. g^{-1} after incubation. The cell count was obtained by serial dilution and plating on BMM. The inoculant was applied to either seeds or seedlings as a slurry of the peat culture and tap water at planting and again one week after planting.

Table 2.3 **(i) Yeast extract mannitol (YEM) agar with Congo red** (Vincent, 1970). The pH of the medium was adjusted to 6.8 to 7.2.

(ii) Yeast extract mannitol-gluconate agar. Based on YEM (Vincent, 1970) and modified by Bhuvaneswari *et al.* (1980).

Table 2.3

(i) Yeast extract mannitol

Chemical	Concentration $g.l^{-1}$
Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
Yeast extract	0.4
NaCl	0.1
Congo red	0.025[a]

[a] 10 ml of 0.25g. 100 ml^{-1} stock solution of Congo red was added to a litre of medium to give a final concentration of 25 ppm

(ii) Yeast extract mannitol-gluconate

Chemical	Concentration g. l^{-1}
Mannitol	5.0
Sodium gluconate	5.0
K_2HPO_4	0.5
Yeast extract	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$CaCl_2.2H_2O$	0.16 *

* 1 ml of this solution was added after autoclaving
The agar concentration for plates was16 g $.l^{-1}$

Table 2.4 **Luria Broth with glucose (LBG)(Miller, 1972).**
The pH of the medium was adjusted to 7.0.

Table 2.4

Chemical	Final concentration g. l^{-1}
Peptone	10
NaCl	15
Yeast extract	5
Glucose	5
Agar	10

Table 2.5 **The composition of Bergersen's Modified Medium (BMM)(Bergersen, 1961).** The pH of the medium was adjusted to 7.0.

Table 2.5

Chemical	Final concentration mg. l^{-1}
$Na_2HPO_4.\ 12H_2O$	360
$MgSO_4.\ 7H_2O$	80
Thiamine-HCl	1
Biotin	0.1
$FeCl_3.\ 6H_2O$	3
$CaCl_2.\ 2H_2O$	40
Sodium glutamate	500
Mannitol	10,000
Yeast extract	500
Gamborg's trace elements *	1 ml $.l^{-1}$

Agar concentration for plates was 16 g. l^{-1}

***Gamborg's trace element solution (Gamborg and Eveleigh,1968)**

$MnSO_4$	10 g $.l^{-1}$
H_3BO_3	3 g. l^{-1}
$ZnSO_4.\ 7H_2O$	3 g. l^{-1}
$Na_2MoO_4.\ 2H_2O$	250 mg. l^{-1}
$CuSO_4.\ 5H_2O$	250 mg. l^{-1}
$CoCl_2.\ 6H_2O$	250 mg. l^{-1}

2.6 Seed surface sterilization

Soybean seeds were surface sterilized in a 100 ml capacity crystallization dish covered with a Petri plate top containing ten 0.5 cm diameter holes for decanting sterilants. After rinsing the seeds in 70 per cent ethanol for 1 minute the seeds were then immersed in 3 per cent commercial sodium hypochlorite for 8 mins. After 8 immediate rinses with sterile distilled water, seeds were transferred to Petri plates containing water agar (1.2 per cent w/v) and germinated in the dark at 28^{o}C. Two days after germination, seedlings with radicals of 0.5 to 1 cm length were transferred to either Leonard jars or plastic growth pouches.

CHAPTER 3

A NEW RECESSIVE GENE CONDITIONING NON-NODULATION IN SOYBEAN

3.1 INTRODUCTION

The genetic contribution of the legume in the establishment of nitrogen-fixing nodules can be determined by the isolation and characterization of symbiotically altered plant mutants. Non-nodulating, ineffectively nodulating and/or supernodulating mutants of legumes are now available in several species including soybean *(Glycine max* (L.) Merr.*)*, chickpea *(Cicer arietinum* L.*)*, peanut *(Arachis hypogaea* L.*)*, pea *(Pisum sativum* L.*)*, red clover *(Trifolium pratense* L.*)* and alfalfa *(Medicago sativa* L.*)* (see Table 1.2). Such mutants altered in nodulation are important in understanding the physiological processes and genetic control of nodule development and function as well as plant growth.

Variants in nodulation have arisen spontaneously either from various geographic accessions or from induced mutagenesis. Naturally-occurring variants which have arisen spontaneously are present in *Trifolium pratense* L. (Nutman, 1949; 1954; 1957; 1968), *Trifolium incarnatum* L. (Smith and Knight, 1984), *Pisum sativum* L. (Gelin and Blixt, 1964; Lie, 1971; Holl, 1975; Lie and Timmermans, 1983), *Medicago sativa* L. (Peterson and Barnes, 1981), *Arachis hypogaea* L. (Gorbet and Burton, 1979; Nigam *et al.,* 1980) and *Glycine max* L. (Williams and Lynch, 1954; Caldwell, 1966; Vest,1970; Vest and Caldwell, 1972; Devine, 1984). Nodulation variants from various geographical accessions exist in *Pisum sativum* L. (Lie, 1971; Young *et al.,* 1982; Kneen and LaRue, 1984b; Lie, 1984). Aberrant nodulation mutants isolated from induced mutagenesis are present in *Cicer arietinum* L. (Davis, 1985; Davis *et al.,* 1985; Davis *et al.,* 1986), *Pisum sativum* L. (Kneen and LaRue, 1984a, Kneen *et al.,* 1986; Jacobsen and Feenstra, 1984), *Medicago sativa* L. (Peterson and Barnes, 1981) and *Glycine max* (L.) Merr. (Carroll *et al.*, 1985a,b; 1986). There are many instances of recessive and dominant inheritance, and cytoplasmic influences involved in nodulation which have been reported in the literature (see Table 1.2). It is interesting to note that all the nodulation variants arising from induced mutagenesis are recessive.

In soybeans five genes affecting symbiotic nitrogen fixation have been documented (Table 1.2). The recessive gene rj_1 (Williams and Lynch, 1954) conditions non-nodulation with all strains of *Bradyrhizobium* tested. However, non-nodulation can be partially circumvented by inoculation with a high cell number of *Bradyrhizobium japonicum* (La Favre and Eaglesham, 1984). The dominant genes Rj_2 , Rj_3 and Rj_4 condition an ineffective nodulation response with certain *Bradyrhizobium* strains (Caldwell, 1966; Vest, 1970; Vest and Caldwell, 1972). Devine (1984) reported a dominant allele in soybean cv. Kent which conditions an ineffective response with the fast growing strain *Rhizobium fredii* USDA205 while a recessive allele carried by the cv. Peking elicits effective nodulation. Three non-nodulation mutants nod49, nod139 and nod772 were isolated by Carroll *et al.* (1986) following induced mutagenesis of the soybean cv. Bragg seeds. A supernodulation and nitrate tolerant symbiosis mutant nts382 which nodulates profusely even in the presence of nitrate was also obtained in the mutagenesis program (Carroll *et al.*, 1985a,b).

This chapter deals with experiments conducted to ascertain whether the mutations in the non-nodulation mutants of soybean are specific to the nodulation process. The mutants were tested for their ability to grow on nitrate as a nitrogen source and to assimilate carbon and nitrogen when grown either with or without a combined nitrogen source. The nature of inheritance of the recently identified non-nodulation characters and the nts382 supernodulation character was determined from the BC_1 progeny and segregation of the BC_1S_1 progeny obtained from crosses between the mutants and the wild-type cv. Bragg. Complementation tests were conducted on the mutants (including rj_1) in order to determine their allelism. Finally, the identification of a new recessive gene is detailed. This gene conditions non-nodulation in nod139 and is tentatively designated as rj_6.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Soybean (*Glycine max* (L.) Merr.) cv. Bragg and its derived non-nodulation mutants nod49, nod139 and nod772 (Carroll *et al.*, 1986), a supernodulation and nitrate tolerant symbiosis mutant nts382 (Carroll *et al.*, 1985a,b) with the naturally-occurring mutation rj_1 (Williams and Lynch, 1954) in the genetic background of the cv. Lee were used in these experiments.

3.2.2 Plant culture

3.2.2.1 Growth studies

In experiments studying different growth parameters, plants were grown in the glasshouse in 15 cm pots filled with a 2 : 1 sand- vermiculite mixture at the rate of one plant per pot for a period of four weeks after germination. The glasshouse temperatures were maintained between 14^{o}C and 30^{o}C. Incandescent bulbs were used to extend the photoperiod to 16 h and to supplement the natural light. Seeds of similar size of all the genotypes were used in these experiments. At planting the seeds were inoculated with a peat culture of *Bradyrhizobium japonicum* strain USDA110 (10^8 cells. pot^{-1}) and the seedlings were reinoculated one week later. Only seedlings which germinated at the same time were used for growth comparisons. Either nitrogen free plant nutrient solution or 6 mM KNO_3 supplemented nutrient solutions were used. The composition of the plant nutrient solution was the same as that used by Herridge (1977). The plants received half strength of all the nutrients (except $CaCl_2$ which was administered at full strength) for the first two weeks after planting and full strength of all the nutrients in the subsequent two weeks before harvest.

Each pot was watered with 1000 ml plant nutrient solution daily which was sufficient to flush out the residual nutrients from the previous watering. Plants were harvested four weeks after germination whereupon their root and shoot dry weights and their nitrogen and carbon content were determined.

3.2.2.2 Genetic studies

Plants were grown under the same conditions as for the growth studies with the exception that they were cultured in larger pots of 25 cm diameter and the glasshouse temperatures were maintained between 14^{o}C and 25^{o}C during the time of cross-pollinating the flowers. Cool humid conditions were maintained in the glasshouse by watering the floor and benches regularly so as to ensure a high percentage of successful cross-pollination . Plants were watered with 5mM KNO_3 supplemented plant nutrient solution three times a week and with tap water on the remaining days. These hybridization experiments were conducted in the southern summer of 1985 and 1986. Flowers were hand emasculated and cross-pollinated between 8a.m. and 10a.m. The cross-pollinated flowers were labelled with a paper tag and the adjoining flowers were removed to enable the crossed flowers to set seeds.

3.2.3 *In vivo* Nitrate Reductase (NR) Assay

In addition to the ability of the soybean mutants to grow on nitrate, their nitrate reductase (NR) activity was assessed to confirm that the mutations were specific to the symbiotic process. *In vivo* NR activity was determined, as described by Carroll and Gresshoff (1986) on unifoliolate leaf discs of 10 day old plants that had been treated either with or without nitrate from planting, using NR assay solution containing 0 or 50 mM KNO_3. The minus NO_3^- (0 mM KNO_3) assay approximates the *in situ* activity wherein the reaction is dependent upon the endogenous pool of metabolically available nitrate within the leaf tissue. The plus NO_3^- (50 mM KNO_3) assay measures the NR enzyme

potential under nitrate-saturated conditions. In both cases, the reactions are dependent upon the *in situ* concentration of cofactors and energy sources (Harper, 1976; Robin *et al.*, 1985). The data were expressed as nmol NO_2^- produced. leaf $disc^{-1}$. h^{-1}.

3.2.4 Nitrogen and Carbon analysis

The percentage of total nitrogen and carbon in the roots and shoots of the plants grown for growth comparisons was determined using a Carlo Erba Carbon and Nitrogen analyser model NA1500. The plant material was oven dried at 80°C and ground thoroughly into a fine powder. Appropriate amounts of plant material (20 mg for roots, 10 mg for shoots and 5 mg for seeds) were weighed for the determination of the carbon and nitrogen content in the samples. Seeds of all the genotypes were collected from plants that had been watered with 5 mM KNO_3 supplemented plant nutrient solution three times a week. Seeds from the same pool were also used for the growth studies. The nitrogen and carbon content of these seeds were also determined.

3.2.5 Crosses and progeny analysis

The non-nodulation mutants nod49, nod139 and nod772 and the supernodulation mutant nts382 were crossed to the wild-type parent cv. Bragg. The F_1 progeny and segregation of the F_2 progeny were observed to determine the mode of inheritance of these characters. Backcrosses were made in reciprocal directions. The progeny were scored for their nodulation phenotypes. Crosses of nod49 and nod772 were also made to a different cultivar, namely, Clark. Reciprocal complementation tests were conducted on nod49, nod139, nod772 and nts382 derived from Bragg, and the naturally-occurring mutation *rj*$_1$ (Lee). The F_1 was observed for nodulation phenotype and the F_2 segregation ratios were recorded for the crosses between all the non-nodulating mutants. In all cases, the F_2 seeds were produced by selfing the F_1 plants in the glasshouse.

The purple hypocotyl and purple flower colour which are present in Clark and rj_1 (Lee) but absent in Bragg and its derivatives were used as a dominant marker in these hybridization programs. Observed segregations for all these studies were tested for goodness-of-fit to expected genetic ratios by Chi-square (X^2) analysis.

3.3 RESULTS

3.3.1 Mutations in nod49, nod772, nod139, rj_1 and nts382 are nodulation specific

Seeds of the soybean genotypes used in these experiments were obtained from plants grown on abundant nitrate but Bragg and particularly nts382 did have some nodules. Under the assumption of a constant nitrogen to protein ratio, the nitrogen content of the seeds of the mutants were extrapolated to give an estimated protein content as detailed in Table 3.1. The genotypes Bragg, nts382, rj_1(Lee) and nod772 had similar nitrogen and carbon content whereas nod49 and nod139 had a slightly lower content of nitrogen. The highest nitrogen content was measured in the seeds of the supernodulating mutant nts382. The percentage of carbon in all the genotypes was similar.

In the absence of nitrate, that is, when the plants were totally dependent on symbiotic nitrogen fixation, the plant dry weight of all the mutants were essentially the same as the wild type (Figure 3.1). However, when the non-nodulating mutants were grown without a combined nitrogen source, the per cent plant nitrogen content in the non-nodulating mutants was lower than in both the nodulating wild-type Bragg and the supernodulation mutant nts382 (Figure 3.2a). A larger discrepency between the non-nodulating mutants and the other genotypes was seen in the shoot than in the root (data not shown). Figure 3.2b shows that the carbon content of all the genotypes was similar. This indicates the absence of anomaly in carbon assimilation in the mutants.

When the genotypes were grown on nitrate, there was a considerable increase in the plant dry weight and percentage plant nitrogen in all the lines. However, the genotypes showed no differences in percentage nitrogen. This was in contrast to the N_2 dependent plants. Furthermore, there was almost a two-fold increase in plant dry matter in all the genotypes. These plant growth comparisons and the accumulation of nitrogen and carbon in both the presence and absence of nitrate indicate that the non-nodulation mutations are nodulation

Table 3.1 **Nitrogen, carbon and protein analyses of the seeds of Bragg, nod49, nod139, nod772, nts382 and *rj*$_1$(Lee).** Plants were cultured with 5 mM KNO_3 supplemented plant nutrient solution administered three times a week. Each entry in the table is the mean of three replications ± S.D.

Table 3.1

Genotype	% Nitrogen	% Carbon	% Protein[a]
Bragg	6.5 ± 0.1	51.9 ± 0.1	40.5 ± 0.0
nod49	5.5 ± 0.3	51.1 ± 0.1	34.6 ± 1.0
nod772	6.5 ± 0.1	50.6 ± 0.0	41.0 ± 0.5
nod139	5.5 ± 0.2	51.8 ± 0.1	34.8 ± 1.0
rj$_1$(Lee)	6.2 ± 0.1	50.4 ± 0.3	38.6 ± 0.6
nts382	6.9 ± 0.1	51.2 ± 0.2	43.1 ± 0.6

[a] estimated from the percentage nitrogen column (conversion factor: % protein estimate = 6.28 x % N)

Seeds were obtained from plants grown in an abundant supply of nitrate

Figure 3.1 **Plant dry weight (P.D.W.) of Bragg, nod49, nod772, nod139, nts382 and *rj*$_1$(Lee).** Plants were cultured either in nitrogen free nutrient solution (- NO_3^-) or 6mM KNO_3 supplemented plant nutrient solution (+ NO_3^-). *Bradyrhizobium japonicum* strain USDA110 (10^8 viable cells. pot^{-1}) was used as the inoculant strain. Plants were harvested four weeks after planting. The data are means of five plants ± S.D.

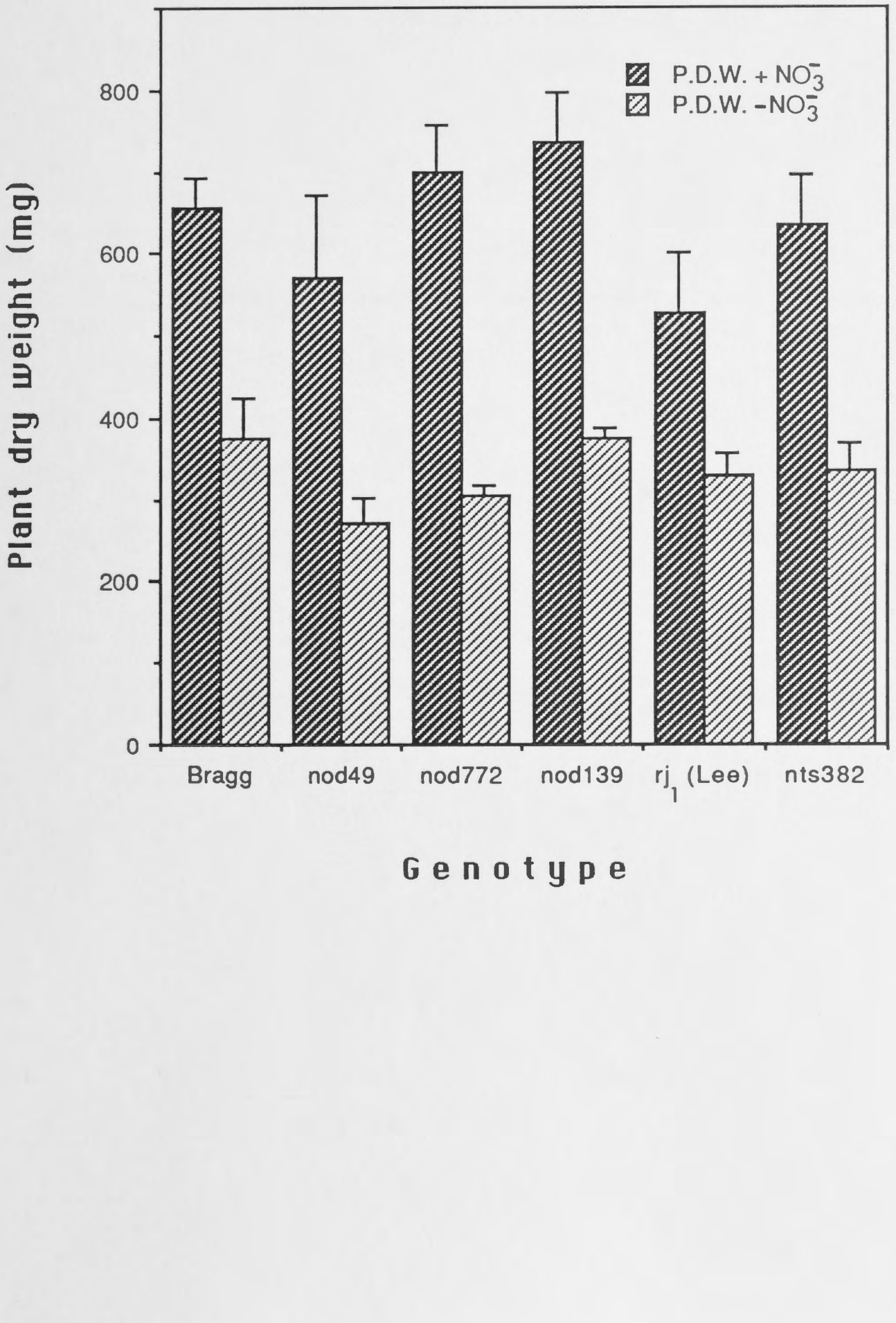
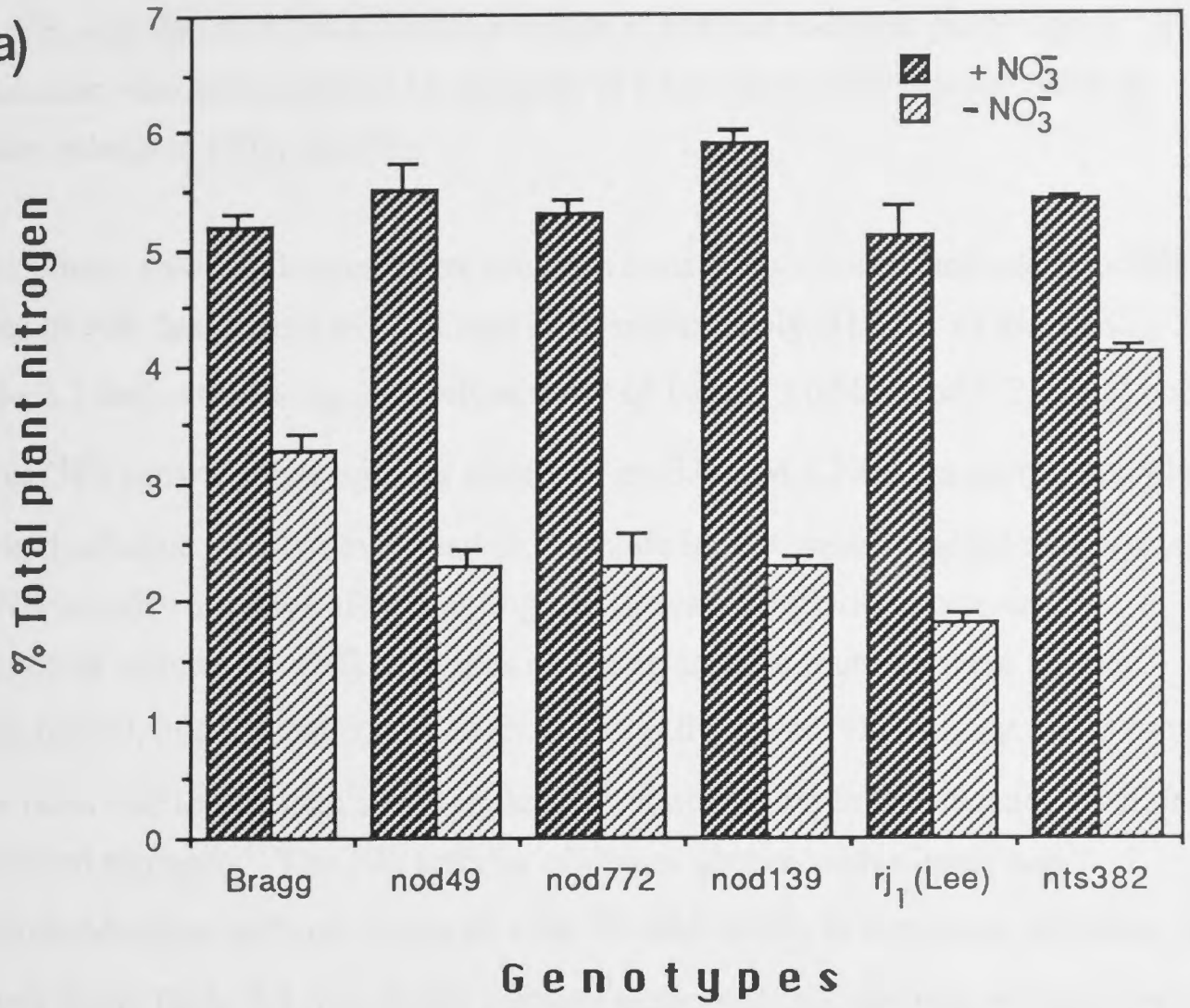

P.D.W. + NO_3^-
P.D.W. -NO_3^-
800
600
400
200
0
Plant dry weight (mg)
Bragg
nod49
nod772
nod139
rj_1 (Lee)
nts382
Genotype

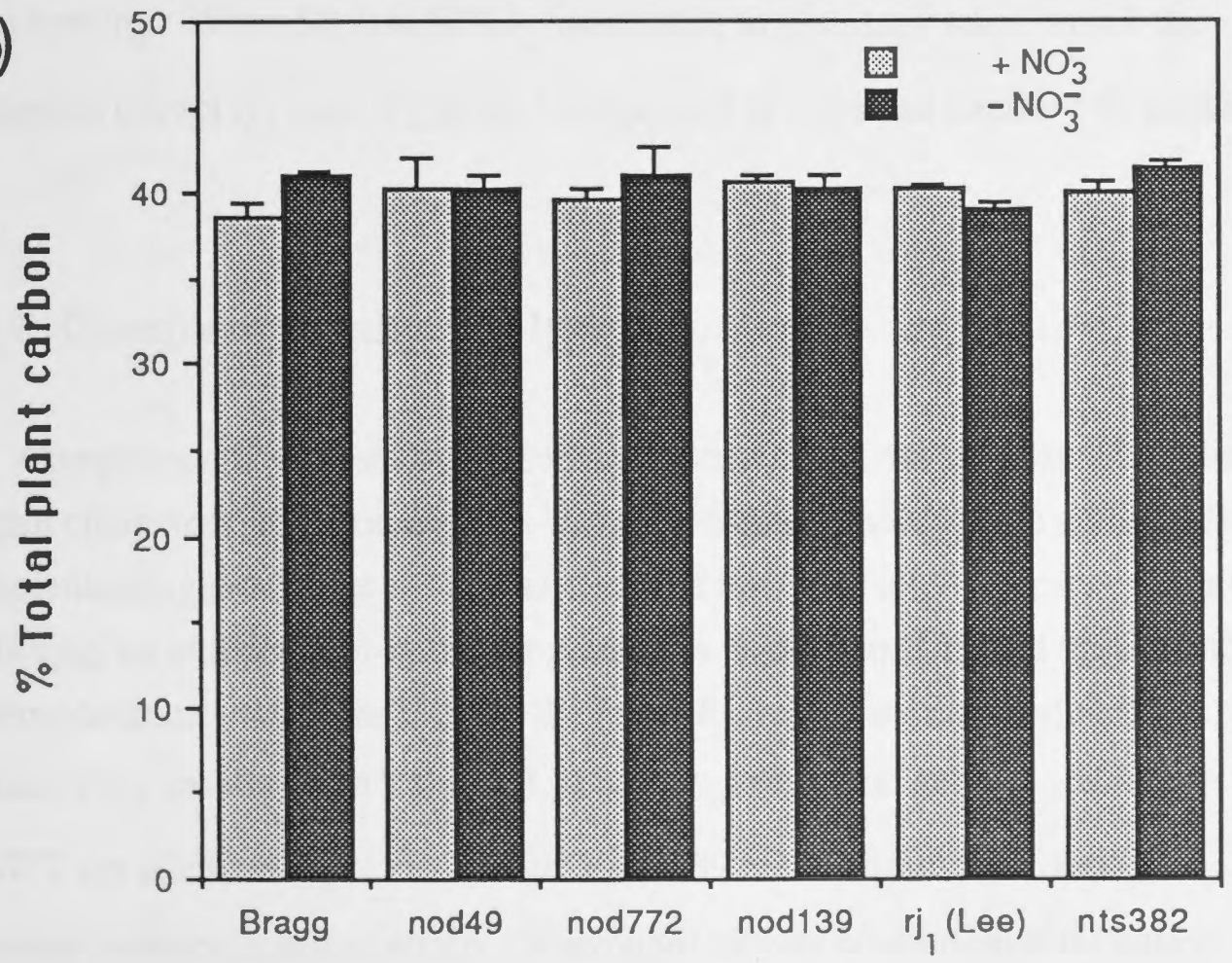
Figure 3.2 **(a) Total plant nitrogen analysis and, (b) total plant carbon analysis for Bragg nod49, nod139, nod772 nts382 and the naturally-occurring non-nodulation mutation *rj1* in the genetic background of the cv. Lee.**

Plants were cultured either with nitrate (+ NO_3^-) or without nitrate (- NO_3^-) as in Figure 3.1. Data are means of five plants ± S.D.

(a)


% Total plant nitrogen
0
1
2
3
4
5
6
7
+ NO3-
- NO3-
Bragg
nod49
nod772
nod139
rj1 (Lee)
nts382
Genotypes

(b)


% Total plant carbon
0
10
20
30
40
50
+ NO3-
- NO3-
Bragg
nod49
nod772
nod139
rj1 (Lee)
nts382
Genotypes

specific, and that their inability to nodulate is not due to lower plant vigour. This conclusion was substantiated by analysis of a biochemical character, such as nitrate reductase (NR) activity.

In young soybean leaves, there are both constitutive and substrate-inducible forms of NR designated as cNR and iNR, respectively (Harper *et al.*, 1985). Table 3.2 indicates the *in vivo* NR activity of Bragg, nod49, nod772, rj_1 (Lee) and nts382 grown either without nitrate or on 3.0 mM $KN0_3$ supplemented plant nutrient solution. Fully developed unifoliolate leaves were sampled and assayed for NR activity ten days after planting. In the case of plants grown in the absence of nitrate, the cNR activities of Bragg and the mutants were similar. Lines nod49, nod139 and rj_1 (Lee) had a slightly lower NR activity. This may have been due to nitrogen stress in these mutants grown in the absence of added combined nitrogen. The NR activity of plants grown with nitrate was determined either without nitrate or with 50 mM $KN0_3$ in the assay solution. It is clear from Table 3.2 that all the mutants expressed iNR activity irrespective of the presence of nitrate in the assay. Bragg and the mutants had essentially the same activity. When 50 mM KNO_3 was added to the assay solution, all the genotypes except rj_1 (in the genetic background of Lee) had similar NR activity.

3.3.2 Complementation analysis

Complementation tests were used to determine allelism because when the mutant characters are recessive, non-complementing genotypes are allelic and complementing genotypes are non-allelic. The results of allelism tests conducted on Bragg, its induced non-nodulating mutants nod49, nod139 and nod772, the supernodulation mutant nts382 and the naturally-occurring non-nodulation mutation rj_1 are shown in Table 3.3. The non-nodulation genes *nod49* and *nod772* are allelic to rj_1 (Lee), whereas *nod139* is not allelic and forms a separate complementation group. When nod139 was crossed with the other non-nodulating mutants, the resultant F_1 was wild type for nodulation. The

Table 3.2 ***In vivo* NR activity of Bragg, nod49, nod772, nod139, rj_1(Lee) and nts382.** Plants were cultured either without nitrate (for constitutive NR assay) or with 3.0 mM KNO_3 supplemented plant nutrient solution (for constitutive plus nitrate-induced NR). Leaf tissue was assayed with either 0 or 50 mM KNO_3 in the assay solution ten days after planting. NR activity is expressed as nmol NO_2 produced. leaf $disc^{-1}$. h^{-1}. Seeds were inoculated at planting with *B. japonicum* strain USDA110. Each entry in the table is the mean of 6 replications ± S.D.

Table 3.2

Genotype	Constitutive NR activity[a]	Inducible plus constitutive NR activity[b]	
		minus NO_3^- assay[c]	plus NO_3 assay[d]
	(n m o l. NO_2^-. leaf disc^{-1}. h^{-1})		
Bragg	21.4 ± 3.6	14.7 ± 4.0	32.4 ± 0.8
nod49	13.6 ± 2.0	13.4 ± 2.0	28.2 ± 5.3
nod772	21.7 ± 4.6	15.3 ± 1.2	29.4 ± 1.2
nod139	17.7 ± 0.1	20.1 ± 1.1	28.7 ± 5.3
rj$_1$(Lee)	15.4 ± 2.0	20.8 ± 9.8	21.1 ± 14.0
nts382	20.3 ± 8.7	20.9 ± 2.0	30.6 ± 5.8

[a] plants were cultured in the absence of nitrate prior to the assay
[b] plants were cultured on nitrate prior to the assay
[c] the NR assay contained 0 mM exogenous nitrate
[d] the NR assay contained 50 mM exogenous nitrate

Table 3.3 **F_1 complementation analysis of the non-nodulation mutants nod49, nod772 and nod139; the supernodulation mutant nts382; and the naturally-occurring non-nodulation mutation *rj1* (Lee).** Plants were scored for allelism six weeks after both germination and inoculation with *B. japonicum* strain USDA110.

Table 3.3

	Bragg	*nod49*	*nod139*	*nod772*	*rj*$_1$(Lee)	*nts382*
Bragg	+	+(12)	+(9)	+(9)	n.d.	+(4)
nod49	+(25)	-	+(7)	-(17)	-(19)	+(9)
nod139	+(21)	+(4)	-	+(9)	+(7)	+(4)
nod772	+(27)	-(30)	+(11)	-	-(17)	+(7)
rj$_1$(Lee)	n.d.	-(15)	+(12)	-(9)	-	n.d
nts382	+(4)	+(11)	+(12)	+(9)	n.d	++

\+ complementation giving normal nodulation
++ supernodulation
\- lack of complementation
n.d. not determined

The numbers in parentheses indicate the number of crosses made in each case

results of reciprocal crosses indicate that no maternal influences are involved in these crosses. Furthermore, the results obtained in Table 3.3 indicate that *nts*382 is recessive and in a separate complementation group distinct from either of the non-nodulation loci.

3.3.3 Inheritance of *nod*49, *nod*139, *nod*772 and *nts*382

Two separate F_1 lines were tested for each of the backcrosses of the non-nodulation mutants nod49, nod139 and nod772 to the parent cv. Bragg. In the case of the supernodulation mutant nts382 only one line was tested to study the inheritance of the mutants. In all the backcrosses to Bragg, the BC_1 progeny had a wild-type pattern of nodulation (Table 3.3) indicating the recessive nature of all the mutants. The BC_1 progenies were selfed and analysed for segregation. The BC_1S_1 progeny segregated into two nodulation phenotypes, wild type and non-nodulation, at a ratio of 3 : 1 (Table 3.4). The non-nodulating progeny were selfed and produced only non-nodulating BC_1S_2 plants. The results obtained thus indicated a monogenic recessive inheritance of nod49, nod139 and nod772 since non-nodulating BC_1S_1 plants were true breeding in the BC_1S_2. The BC_1S_1 progenies of the backcross of nts382 to Bragg segregated into wild-type and supernodulation phenotypes at a 3 : 1 ratio. This result indicates a monogenic recessive inheritance for this mutation (Table 3.4). Similar results were obtained with the crosses of both nod49 and nod772 to cv. Clark, a different genetic background to that from which the mutants were isolated (Table 3.5). The dominant purple hypocotyl and purple flower colour in Clark served as a marker since Bragg and its mutants lacked this character. The purple hypocotyl colour in Clark segregated independently of the non-nodulation characters as indicated by X^2 values for the expected segregation ratios of 9 : 3 : 3 :1 in the F_2 (Table 3.5). The F_2 non-nodulating progeny produced only true breeding non-nodulating progeny again indicating a monogenic recessive inheritance of *nod*49 and *nod*772 when also crossed with the cv. Clark. Crosses between nod139 and Clark failed to produce hybrid seeds.

Table 3.4 **Monogenic recessive inheritance of non-nodulation in soybean mutants nod49, nod772 and nod139 and supernodulation in nts382.** The mutants were crossed to the parent cv. Bragg and the BC_1S_1 segregation ratios were determined. The observed and the expected (3 : 1) ratios along with the calculated X^2 values are listed.

Table 3.4

Cross (♀ x ♂)	BC_1S_1 segregation ratios Observed		Expected		Calculated X^2 (3 : 1)
	wild type	non-nodulating	wild type	non-nodulating	
nod49 x Bragg					
1.	98	42	105.00	35.00	1.867*
2.	105	34	104.25	34.75	0.022*
nod772 x Bragg					
1.	107	23	97.50	32.50	3.703*
2.	126	38	123.00	41.00	0.293*
nod139 x Bragg					
1.	148	47	146.25	48.75	0.084*
2.	101	35	102.00	34.00	0.039*
	wild type	supernodulating	wild type	supernodulating	
nts382 x Bragg	165	60	168.75	56.25	0.333*

* all calculated X^2 values for the BC_1S_1 progeny segregation were not significantly different from the expected ratio (3:1) at the 5 per cent critical value; tabulated X^2 for one degree of freedom is 3.84

Table 3.5 **Monogenic recessive inheritance of non-nodulation in soybean mutants nod49 and nod772.** The mutants were crossed to the cv. Clark. The F_2 segregation ratios for non-nodulation were determined. Purple hypocotyl and purple flower colour in Clark segregated independently of the non-nodulation character at a 9 : 3 : 3 : 1 ratio. The observed ratios were tested for goodness-of-fit to the expected ratios by Chi-square analysis. The calculated X^2 values are listed.

Table 3.5

F_2 segregation for nodulation and purple hypocotyl colour in cv. Clark

Cross (♀ x ♂)	Purple hypocotyl		White hypocotyl		Calculated X^2
	Nodulating	Non-nodulating	Nodulating	Non-nodulating	
Cross No.	observed expected	observed expected	observed expected	observed expected	(9:3:3:1)
nod49 x Clark					
1. 183	169.875	56 56.625	52 56.625	11 18.875	4.685*
2. 82	69.750	19 23.250	15 23.250	8 7.750	5.864*
nod772 x Clark					
1. 34	33.188	9 11.063	11 11.063	5 3.688	0.872*
2. 36	41.625	15 13.875	18 13.875	5 4.625	2.108*

* all calculated X^2 values were not significantly different from the expected ratio (9:3:3:1) at the 5 per cent critical level; the tabulated X^2 for 3 degrees of freedom is 7.82.

The F_2 segregation ratios of the progenies obtained from crosses between nod139 and the other non-nodulation mutants (allelic to *rj*$_1$) were examined to study the linkage between the loci. The Chi-square was significant at both the 9 : 7 and 1 : 1 ratio (Table 3.6). The F_2 progenies of the crosses between nod49 and nod772 when crossed to nod139 segregated for the wild-type and non-nodulating phenotypes. The F_2 segregation of the crosses between nod49 and nod772 (both allelic to *rj*$_1$) produced true breeding plants (Table 3.6). In all cases, two F_1 plants from two distinct crosses were used to study the F_2 segregation. The results obtained in Table 3.7 show that the F_2 segregation of the crosses was statistically significant at both the 6 : 6 : 2 : 2 and the 27 : 21 : 9 : 7 ratio, being the expected ratio for tight linkage and independent segregation of *rj*$_1$ and *rj*$_6$, respectively. The purple hypocotyl colour in Lee *(rj*$_1$*)* and Clark segregated independently of the non-nodulation characters at the expected ratios (Table 3.7 and 3.8).

In all the crossing programs involving the mutants, the F_2 segregation produced a small proportion of the progenies which had deformed leaves which were confined to the initial five trifoliolate leaf stage. These were termed 'leaf mutants'. Some of the others were stunted plants and had only cotyledons and were called 'dwarves'. These may have been due to other mutations in the plant material. These mutants segregated at a ratio of 1 mutant : 3 normal indicating they are probably a genetic phenomenon. These segregants were not included in the results presented here because the nodulation of the phenotypes were sometimes difficult to distinguish due to decreased vigour.

Table 3.6 **Segregation of nodulation phenotypes in the F_2 progenies from crosses between the induced non-nodulation mutants obtained from the soybean cv. Bragg.** The observed ratios of the crosses between nod49 and nod772 crossed to nod139 were tested for goodness-of-fit to the expected ratios by Chi-square analysis. The calculated X^2 values for the two ratios (9 : 7 and 1 : 1) are listed.

Table 3.6

Cross (♀ x ♂)	Cross No.	Observed ratio wild-type	non-nodulating	Expected ratio wild-type	non-nodulating	Calculated X^2 9 : 7[a]	1 : 1[b]
nod49 x nod139	1. 17	11		15.750	12.250	0.2268	1.286
	2. 20	14		19.125	14.875	0.092	1.058
nod139 x nod49	1. 26	17		24.188	18.813	0.311	1.885
	2. 16	15		17.438	13.653	0.271	0.032
nod772 x nod139	1. 29	20		27.563	21.438	0.071	1.654
	2. 24	14		21.375	16.625	0.737	2.632
nod139 x nod772	1. 22	19		23.063	17.938	0.112	0.220
				0 : n[c]		0 : n[c]	
nod49 x nod772	1. 0	32		0.000	32.000	0.000	
	2. 0	27		0.000	27.000	0.000	
nod772 x nod49	1. 0	36		0.000	36.000	0.000	
	2. 0	45		0.000	45.000	0.000	

[a,b] Calculated X^2 values for F_2 segregation for nodulation were not significantly different from the expected ratios (9:7 and 1:1) at the 5 per cent critical value; tabulated X^2 value for one degree of freedom is 3.84

[c] All progeny were non-nodulating, thus the calclated X^2 was consistently 0 and hence highly significant

Table 3.7 **Linkage possibilities between the naturally-occurring mutation *rj*$_1$ (Lee) and the induced non-nodulation mutant nod139.** The purple hypocotyl and purple flower colour (*W*$_1$) in Lee was used as a marker.

Table 3.7

Cross (♀ x ♂)	Linkage possibilities				
			Total linkage		
	$W_1 rj_1$ [a]	$W_1 rj_6$ [b]	$W_1 rj_1 rj_6$ [c]	$rj_1 rj_6$ [d]	No linkage[e]
nod139 x rj_1(Lee)	1. 9.736^{ns}	23.707^{ns}	23.707^{ns}	2.706*	0.227*
	2. 28.957^{ns}	25.706^{ns}	25.706^{ns}	1.335*	5.810*
rj_1(Lee) x nod139	1. 27.482^{ns}	20.069^{ns}	20.069^{ns}	0.899*	3.265*
	2. 10.165^{ns}	25.352^{ns}	25.352^{ns}	0.752*	4.473*

* calculated X^2 values for F_2 segregation were not significantly different from the expected ratios; tabulated X^2 value for 3 degrees of freedom is 7.82

^{ns} calculated X^2 values for F_2 segregation were significantly different from the expected ratios

[a] linkage of purple hypocotyl and flower colour to rj_1

[b] linkage of purple hypocotyl and flower colour to rj_6

[c] linkage of purple hypocotyl and flower colour to rj_1 and rj_6

[d] total linkage of rj_1 and rj_6

[e] no linkage of rj_1, rj_6 and purple hypocotyl and flower colour

Table 3.8 **F_2 segregation of progeny derived from crosses between the naturally-occurring non-nodulation mutation *rj1* (in the genetic background of the cv. Lee) and the induced non-nodulation mutants of the soybean cv. Bragg.** Lee carried a purple hypocotyl and flower pigmentation which was used as a marker. Purple colour segregated independently of the non-nodulation characters at a 3 : 1 ratio. Chi-square analysis was used to test the goodness-of-fit of the observed ratios with the expected ratios.

Table 3.8

F_2 segregation for nodulation and purple hypocotyl colour in Clark

	Purple hypocotyl			White hypocotyl				Calculated	
	Nodulating		Non-nodulating	Nodulating		Non-nodulating		X^2	
	O	E	O	E	O	E	O	E	(27:21:9:7)(3 : 1)	
Cross No.				**nod139 x *rj*$_1$(Lee)**						
1.	73	75.52	59	58.73	27	25.17	20	19.58	0.227*	-
2.	77	84.79	74	65.95	21	28.27	29	21.98	5.210*	-
				***rj*$_1$(Lee) x nod139**						
1.	65	69.61	59	54.14	18	23.20	23	18.05	3.264*	-
2.	44	50.20	49	39.05	13	16.73	14	13.05	4.473*	-
				nod49 x *rj*$_1$(Lee)						
1.	0	0	32	29.25	0	0	9	9.75	-	*
2.	0	0	30	33.00	0	0	14	11.00	-	*
				nod772 x *rj*$_1$(Lee)						
1.	0	0	78	75.75	0	0	23	25.25	-	*
2.	0	0	32	33.00	0	0	12	11.00	-	*

* all calculated X^2 values were not significantly different from the expected ratios (27:21:9:7 and 3:1) at the 5 per cent critical value; tabulated X^2 for 3 and 1 degree of freedom are 7.82 and 3.84, respectively

Lee carried a purple hypocotyl and flower pigmentation which was used as a marker

O - observed
E - expected

3.4 DISCUSSION

The results presented in this chapter indicate that the induced non-nodulation mutants nod49, nod139 and nod772 along with the naturally-occurring non-nodulation mutation rj_1 in the genetic background of the cv. Lee are not altered in the assimilation of nitrogen and carbon when grown on nitrate (Figures 3.1 and 3.2). Weber (1966) obtained similar results for field tests of rj_1 plants, where increased applied nitrogen produced increased seed and dry matter yields as well as increased seed size and protein content. The supernodulation mutant nts382 which is a nitrate tolerant symbiosis mutant and nodulates profusely in the presence of nitrate (Carroll *et al.*, 1985a,b) is also unaltered in the assimilation of nitrate. A similar conclusion was drawn by Carroll *et al.* (1985a,b) and Day *et al.* (1986). Since nitrogen contents were only substantially lower in the non-nodulating mutants in the absence of combined nitrogen (Figure 3.2a), the anomaly in the non-nodulation mutants is clearly specific to the nodulation process. This was confirmed by assaying NR (an enzyme not directly related to nodulation) under a wide range of conditions. The non-nodulating mutants expressed cNR and iNR activity similar to Bragg (Table 3.2). In a more general context, increases in nitrogen content in Bragg and nts382 in the presence of nitrate indicate that seed reserves and symbiotic nitrogen fixation (which is dependent on nodule formation) in these genotypes could not provide all the nitrogen required by these plants, thus emphasising the importance of a combined source of nitrogen during early development (Harper, 1974).

The non-nodulation character in mutants nod49, nod139 and nod772 and the supernodulation trait in mutant nts382 are inherited as Mendelian monogenic recessives as seen in crosses to the parent cv. Bragg (Table 3.4). Mutants nod49 and nod772 crossed to the cv. Clark gave similar results (Table 3.5). Non-nodulation in rj_1 (Williams and Lynch, 1954) is inherited as a monogenic recessive while ineffective nodulation in Rj_2, Rj_3 and Rj_4 (Caldwell, 1966; Vest,1970; Vest and Caldwell, 1972) are inherited as monogenic dominant loci.

Like all reported induced mutations affecting nodulation *nod*49, *nod*772 and *nod* 139 are recessives indicating that some function required for nodulation is anomalous in these mutants. Previous to this study, the recessive rj_1 allele had been reported to have occurred only once in the U.S. breeding lines, suggesting that the allele represents a solitary mutation event (Devine and Breithaupt, 1980). Attempts have been made to explain the occurrence of nodulation variants in legumes, especially the *Rj* genes. While studying the frequency of Rj_2 and Rj_4 gene distribution in Asia, Devine and Breithaupt (1980) found that aberrant nodulation response in these genes is associated with their geographic distribution and is not a phylogenetically recent mutation event. Incompatible effects could have resulted from the introduction of host germplasm to areas where the association of the host and rhizobia may have been alien to each other. It is interesting to note that all the geographical variants of soybean are dominant. In contrast, the geographical variants in pea are usually recessive. This difference could be due to likely genome duplication in soybean (Crane *et al.*, 1982) since recessive variations would be masked if a locus is duplicated. Presumably, the non-nodulation genes of soybean affected by induced mutation in nod49, nod772 and nod139 do not have duplicate wild-type alleles.

Complementation tests conducted on *nod*49, *nod*139, *nod*772, *nts*382 and rj_1 (Lee) indicate that the mutants nod49, nod772 and rj_1(Lee) are allelic. On the other hand, the results in Table 3.3 indicate that *nod*139 is not allelic to either rj_1 (Lee), *nod*49 or *nod*772 and is defective in a separate function required for nodulation (Table 3.3). The gene symbol rj_6 is tentatively proposed for the new recessive gene which conditions non-nodulation in soybeans. The nomenclature of soybean genes affecting nodulation is inadequate and now appears to be obsolete. The symbol *Rj* is now inappropriate since the bacteria nodulating soybeans is no longer called *Rhizobium japonicum* rather *Bradyrhizobium japonicum* or *Rhizobium fredii*. A more appropriate symbol is required which should be distinct for the non-nodulating and ineffective genes. The supernodulation character of nts382 is determined by a locus different from rj_1 and the new non-nodulation gene rj_6 present in nod139, and therefore

requires a separate designation.

The phenotypes of the non-nodulating mutants nod49 and nod772 which are allelic to rj_1 demonstrate that they came from independent mutation events (Mathews *et al.*, 1987a). The occurrence of the separate mutations indicate a 'hot-spot' for mutation at the rj_1 locus or nearby loci such that complementation does not occur between the mutations. A hot-spot for mutation in *Pisum sativum* has also been reported at the *sym*-5 locus (Kneen *et al.*, 1986). The existence of physically close genes which confer resistance to disease have been reported in plant-disease interactions. On chromosome 5 of barley and chromosome 2 of wheat, some genes for resistance to several diseases appear to be physically close (Day *et al.*, 1983). Therefore nodulation neighbourhoods may exist within the legume genome in a similar way to the organization of the leghemoglobin genes and pseudogenes (Bojsen *et al.*, 1985). Genetic linkage has been established between the rj_1 locus controlling non-nodulation and the *F* locus controlling fasciated stem in soybean (Devine *et al.*, 1983b). The two loci are separated by a distance of 40 ± 2.2 genetic map units on linkage group 11. In mutants nod49, nod139 and nod772 it was not possible to ascertain whether the *nod139* (rj_6) mutation is linked to rj_1 since the segregation ratios in the F_2 closely resembled that expected for both tight linkage and independent segregation (Table 3.6 and 3.7). Thus, the absence of any known morphological markers linked to either rj_1 or rj_6 in these mutants makes the study of linkage in these mutants inconclusive. The dominant purple flower marker present in rj_1 (cv. Lee) is not linked to either rj_1 or rj_6. Devine *et al.* (1983b) also observed that the rj_1 locus was independent of the W_1 locus (purple flower colour) in linkage group 8. The genetic map of soybean is very incomplete due to the large number of chromosomes ($2n = 40$) and the inadequate number of morphological markers. These problems are exacerbated by the difficulty in cross-pollinating soybeans which have small, self-pollinating flowers. In addition, each successful cross-pollination produces at best only three to four seeds.

In summary, the results reported in this chapter indicate that the non-nodulation mutants nod49, nod139 and nod772, the naturally-occurring non-nodulation mutation rj_1 (Lee) and the supernodulation mutant nts382 are not altered in the nitrogen and carbon assimilation when grown on soil nitrate. The non-nodulation characters are inherited as Mendelian monogenic recessives. The non-nodulating mutant nod139 is not allelic to rj_1 and the proposed symbol for this new recessive gene conditioning non-nodulation in nod139 is rj_6.

CHAPTER 4

CHARACTERIZATION OF ALTERED NODULATION MUTANTS OF SOYBEAN [*Glycine max* (L.) Merr.]: *Bradyrhizobium* EFFECTS AND THE ABSENCE OF ROOT HAIR CURLING

4.1 INTRODUCTION

Symbiotic nitrogen fixation in legumes is a complex multi-step process involving both the leguminous plants and the rhizobia. Genetic factors which are inherited separately in the two partners together with the environment interact to produce a joint phenotype - the nitrogen fixing root nodule. Non-nodulation mutants or variants have been identified in several legume species.

Williams and Lynch (1954) reported a nodulation-resistant character in soybeans (*Glycine max* [L.] Merr.) conditioned by a single recessive gene (rj_1). The naturally-occurring non-nodulation soybean mutant rj_1 had been screened for nodulation using a number of *Bradyrhizobium* strains either singly or pooled into inoculant mixtures. No nodulation was observed in soil cultures but occasional and irregular nodulation was evident in sand-nutrient solution cultures. The reisolated rhizobia from these nodules did not produce a wild-type nodulation pattern (Clark, 1957). La Favre and Eaglesham (1984) observed an increase in nodulation of the rj_1 soybeans after inoculation with high cell numbers.

Despite the fact that more than thirty years have passed since the original discovery of the rj_1 soybean, the exact blockage in symbiotic development for either the rj_1 mutant or other non-nodulation mutants reported in other legumes has not been determined. This study reports the initial characterization of the non-nodulation mutants nod49, nod139 and nod772 and the supernodulation mutant nts382 as well as the further characterization of the naturally-occurring rj_1 mutation for root hair related infection events and microbiological conditionality. Root hair characteristics, the effect of various *Bradyrhizobium* strains, the effect of different doses of inoculation on the nodulation of the mutants and attempts to circumvent non-nodulation are also described in this chapter.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Non-nodulation mutants nod49, nod139 and nod772, the supernodulation mutant nts382, the parent cultivar Bragg and the *rj*$_1$ mutation in the genetic background of the cv. Lee were used for the experiments in this chapter. The isolation procedures of the mutants nod49, nod139, nod772 and nts382 have been described in detail by Carroll *et al.* (1985a,b; 1986). Mutant nod49 was obtained from a mixed M_2 population screened in the field whereas mutants nod139 and nod772 were isolated from segregating M_2 families derived from EMS mutagenesis (Carroll *et al.*, 1986).

4.2.2 Plant culture

In experiments studying the dose and strain effects on nodulation, plants were cultured in either Leonard jars or magenta jars using vermiculite and half strength Herridge's N-free nutrient solution (Herridge, 1977). Seeds of Bragg were surface sterilized as described in Section 2.6 in Chapter 2. Germinated seedlings having radicals of 0.5 to 1 cm length were transferred to Leonard jars and inoculated with various *Bradyrhizobium japonicum* strains at different inoculant doses. Uninoculated jars were used as controls. Plants were cultured in partially temperature-controlled glasshouses with a temperature range of 15 - 30^{o}C. Nodule numbers and nodule fresh weights were noted at the time of harvest. Nodule and plant dry weights were obtained after oven drying the samples. To study the effect of cotyledon removal, cotyledons were removed a week after germination. For studies which involved the addition of small

amounts of nitrate, 0.5 mM KNO_3 was added to the Leonard jar nutrient solution.

Root hair characteristics and lateral roots were studied by growing the seedlings in 20 cm diameter pots containing vermiculite. Germinated seeds were inoculated with *Bradyrhizobium japonicum* strain USDA110 at 10^8 viable cells. $seed^{-1}$. One week and two week old seedlings were used to determine lateral root numbers and the percentage of curled root hairs. Only markedly curled root hairs were noted.

Plants were vegetatively propagated by cuttings at one week after germination and were placed in potting mixture and maintained in a mist chamber for 2 weeks to enable the rooting of these cuttings. The rooted cuttings were then transferred to the greenhouse and observed for nodulation.

4.2.3 *Bradyrhizobium* and *Rhizobium* strains

Sixteen strains of *Bradyrhizobium japonicum* obtained from Mr. John Brockwell (Plant Industry, C.S.I.R.O., Canberra) were used in the experiments along with other *B. japonicum* strains. In addition, *Bradyrhizobium Parasponia* strain ANU289 and the cowpea strains CB756, 32HI and NGR234 were used. Seeds were inoculated with low (10^6-10^7 viable cells. Leonard jar^{-1}), medium (10^7-10^8 viable cells. Leonard jar^{-1}) and high (10^8-10^9 viable cells. Leonard jar^{-1}) inoculant doses. The bacterial cultures were grown to mid-logarithmic phase in yeast extract mannitol broth and serially diluted to obtain the required cell numbers. Viable cell numbers were determined by dilution platings on yeast extract mannitol agar medium. In experiments involving the effect of combination of *Bradyrhizobium japonicum* strains, the strains were pooled to form inoculant mixtures and were subsequently used to inoculate the germinated seeds in Leonard jars. Some results were confirmed in smaller volume plastic magenta jars.

Rhizobia were reisolated from occasional nodules on nod49 by detaching the nodule along with a portion of the root. The nodule was then surface sterilized with 0.1 per cent (w/v) mercuric chloride for 5 min followed by rinsing with sterile distilled water to remove the mercuric chloride. This was

followed by immersion in 70 per cent ethanol for 3 minutes. The nodule was rinsed several times with sterile distilled water and crushed in a test tube containing about 5 ml sterile water using a sterile glass rod. The supernatant was streaked on yeast extract mannitol agar plates containing Congo red and the plates were incubated at 28^{o}C. Isolated colonies were selected and transferred to agar slants. The reisolated rhizobia were tested on the non-nodulation mutants using the Leonard jar assembly.

4.2.4 Acetylene reduction assay

Nitrogenase activity was estimated by measuring acetylene reduction. Decapitated root systems were placed in 1 litre gas-tight jars fitted with serum stoppers and 50 ml acetylene was added. The jars were then incubated at room temperature for 20 mins. Gas samples (0.2 ml) were removed for analysis by gas chromatography. Nitrogenase activity was determined from the rate of ethylene produced.

4.2.5 Statistical analysis

Data were analysed by the GENSTAT statistical package (Alvey *et al.*, 1977).

4.3 RESULTS

4.3.1 Effect of *Bradyrhizobium* and *Rhizobium* strains on nodulation

Experiments were conducted using a broad genetic diversity of rhizobia as inoculants in order to study their effects on nodulation and growth of the mutants. Bragg, rj_1(Lee), nod49, nod139, nod772 and nts382 were tested for nodulation with several *Bradyrhizobium japonicum* strains. Native *Glycine Bradyrhizobium* strains CC1601b (ex. *Glycine clandestina*) CC1602d (ex. *G. tabacina*), CC1603d (ex. *G. tomentella*) and CC1604b (ex. *G. canescens*)were used along with cowpea strains NGR234, 32HI and CB756 and *Parasponia Bradyrhizobium* ANU289. Several *Rhizobium fredii* strains were also tested. Plants were cultured in Leonard jars for 4 weeks. Non-nodulation mutants nod49 and nod139 failed to nodulate at low inoculant levels. This is consistent with the nodulation observations on nod49 and nod139 plants grown in soil at the Breeza Experimental Station in New South Wales, Australia (D. Herridge, *pers. comm.*) and at an Agrigenetics farm site in Wisconsin, USA (E. Appelbaum, *pers. comm.*). However, mutant nod49 formed a few nodules with some *Bradyrhizobium japonicum* and some *R. fredii* strains at high levels of inoculation. For example, the mean nodule number per plant was 2 with *R. fredii* CC736 (=USDA192) and 8 with *B. japonicum* CB1795 (=USDA96) at high cell numbers (10^8-10^9 viable cells. plant^{-1}). Table 4.1 shows the nodulation on Bragg and the mutants induced by different cell numbers of *B. japonicum* strain USDA1795. Nodules were formed on the non-nodulation mutants at medium (10^7 viable cells. ml^{-1}) and high (10^9 viable cells .ml^{-1}) inoculant doses. Nodule formation was consistently higher in nod772 than in the other non-nodulation mutants. Nodulation on nts382 increased when inoculated with medium and high cell numbers of *B. japonicum* strain CB1795 when compared to the low inoculation treatment. On the other hand, nodulation on Bragg did not vary significantly when the inoculant cell numbers were varied.

Table 4.1 Effect of different inoculant cell numbers on the nodulation of Bragg, the non-nodulation mutants nod49, nod772, nod139 and rj_1(Lee) and the supernodulation mutant nts382. Plants were grown in Leonard jars either uninoculated or inoculated with 1 ml of *Bradyrhizobium japonicum* strain CB1795 at low (1×10^5 viable cells. ml^{-1}), medium ($1x\ 10^7$ viable cells. ml^{-1}) and high (1×10^9 viable cells. ml^{-1}). Each entry in the table is the mean of 4 plants ± S.D.

Table 4.1

Treatment	Nodule number . plant^{-1}	Nodule fresh weight (mg)	Nodule dry weight (mg)	Plant dry weight (mg)	Shoot/ root	nmol C_2H_4 . mg. .NFW^{-1}
1. Uninoculated						
Bragg	0	0	0	482 ± 324	2.45	0
nod49	0	0	0	386 ± 23	3.11	0
nod772	0	0	0	672 ± 272	3.11	0
nod139	0	0	0	266 ± 83	2.76	0
rj$_1$(Lee)	0	0	0	407 ± 70	2.63	0
nts382	0	0	0	633 ± 178	3.03	0
2. Low inoculant dose (1 x 10^5 viable cells. ml^{-1})						
Bragg	25 ± 3	220 ± 19	50 ± 3	624 ± 74	2.66	121 ± 39
nod49	0	0	0	361 ± 43	2.92	0
nod772	0	0	0	531 ± 218	2.45	0
nod139	0	0	0	349 ± 59	2.73	0
rj$_1$(Lee)	2*	n.d.	n.d.	412 ± 61	2.35	0
nts382	165 ± 35	520 ± 99	92 ± 10	508 ± 33	2.66	43 ± 21
3. Medium inoculant dose (1 x 10^7 viable cells. ml^{-1})						
Bragg	28 ± 6	240 ± 69	42 ± 29	466 ± 202	2.46	181 ± 23
nod49	2*	17*	4*	265 ± 72	2.64	405*
nod772	11 ± 6	12 ± 6	5 ± 4	319 ± 145	2.98	176 ± 84
nod139	5*	6*	2*	282 + 105	2.79	56*
rj$_1$(Lee)	10 ± 3	81 ± 4	14 ± 3	347 ± 37	2.20	164 ± 57
nts382	391± 44	760 ± 27	109 ± 8	307 ± 22	5.79	37 ± 21
4. High inoculant dose (1 x 10^9 viable cells. ml^{-1})						
Bragg	28 ± 5	171 ± 38	32 ± 13	442 ± 135	2.15	166 ± 11
nod49	1.5**	10**	4**	293 ± 16	2.10	60**
nod772	11 ± 5	15 ± 4	3 ± 1	343 ± 45	2.15	41 ± 0
nod139	3 ± 2	18 ± 8	3 ± 1	440 ± 67	2.04	68 ± 0
rj$_1$(Lee)	10 ± 3	45 ± 22	9 ± 4	428 ± 69	1.87	109 ± 0
nts382	286 ± 102	692 ± 365	107 ± 53	147 ± 62	5.98	40 ± 24

* a nodule on one out of 4 plants
** a nodule on two out of 4 plants
n.d. not determined

In a separate experiment, it was observed that the nodulation frequency, expressed as percentage of plants nodulated, increased in nod49 when the inoculant titre was increased from 10^7cells. plant^{-1} to 10^{12} cells. plant^{-1}. High inoculant titres were obtained by concentrating the cells by centrifugation. Inoculation of 10^{12} rhizobia. plant^{-1} resulted in 100 per cent nodulation of the plants. Increasing the cell numbers resulted in a higher nodule number per mutant plant, even so this was still very low at the highest inoculant dose. Mutant nod49 formed no nodules with any of the native *Glycine Bradyrhizobium* strains tested both at medium and high dose of inoculation. Bragg nodulated with native *Glycine Bradyrhizobium* CC1601b, CC1602d and CC1603d at the high dose of inoculation. Strain CC1604b formed a mean of 5 nodule-like structures at high dose inoculation on Bragg. Likewise, the non-nodulating mutants did not form any nodules with either any of the cowpea *Bradyrhizobium* strains or *Parasponia Bradyrhizobium* ANU289. These strains nodulated the parent cultivar Bragg either very poorly or not at all.

4.3.2 Root hair characteristics and lateral root numbers

Bragg, nod49, nod772, nod139, rj_1(Lee) and nts382 were studied for root hair characteristics one week and two weeks after planting and inoculation with *Bradyrhizobium japonicum* strain USDA110 at the medium inoculant dose. The terminal 8 cm of the tap root was excised, stained with toluidine blue and mounted on a slide using glycerol. Root hairs were scanned on both the left and right hand side of the roots. Only markedly curled (>360^o) root hairs were recorded. The results obtained are shown in Table 4.2. The total number of root hairs in this region of the root decreased between 1 week and 2 weeks while the percentage curled root hairs in Bragg, nts382 and nod772 increased from 10.1 per cent to 11.7 per cent, 10.9 per cent to 15.8 per cent and 0.6 per cent to 2.3 per cent, respectively. Mutants nod49, nod139 and rj_1(Lee) had no markedly curled root hairs at either sampling. Figure 4.1 shows the root hairs on a nod49 root.

Lateral root numbers for Bragg, nod49 and nod139 were not significantly

Table 4.2 **Total root hairs, per cent root hairs and lateral root numbers for Bragg, nod49, nod772, nod139, *rj*$_1$(Lee) and nts382.** Markedly curled root hairs on the left and right hand side of the terminal 8 cm of the tap root were counted after staining the roots with toluidine blue. Each entry in the table is the mean of 5 to 8 plants. Lateral root numbers were observed a week (average of 6 plants) and two weeks (average of 9 plants) after inoculation. Germinated seeds were inoculated at planting with *B. japonicum* strain USDA110 at 10^8 viable cells. seed^{-1}.

Table 4.2

Plant	Total root hairs		% curled root hairs		Lateral root numbers	
			Weeks after inoculation			
Genotype	1 week	2 weeks	1 week	2 weeks	1 week	2 weeks
Bragg	2220	1543	10.1	11.7	59.8	67.3
nod49	1450	1072	0	0	56.1	77.6
nod772	735	412	0.6	2.3	71.8	79.2
nod139	1732	288	0	0	58.0	75.8
rj$_1$(Lee)	704	265	0	0	80.5	83.6
nts382	1543	537	10.9	15.8	81.2	91.1
L.S.D.	-	-	-	-	6.4	8.2

Figure 4.1 **Root hairs on nod49 root.** Seeds were inoculated at planting with *B. japonicum strain* USDA110 at medium (1×10^8 viable cells. ml^{-1}) dose. Note the absence of marked root hair curling

different from each other one week after germination while mutant nod772 had a higher number of lateral roots. The *rj*$_1$ mutant had a significantly higher number of lateral roots compared to the remaining non-nodulation mutants at one week old stage. Two weeks after germination, there were no significant differences in the lateral root numbers of all the non-nodulation mutants: *rj*$_1$(Lee), nod49, nod139 and nod772. In contrast, the supernodulation mutant nts382 had a higher number of lateral roots compared to the wild-type Bragg as shown in Table 4.2. These results confirmed those of Day *et al.* (1986).

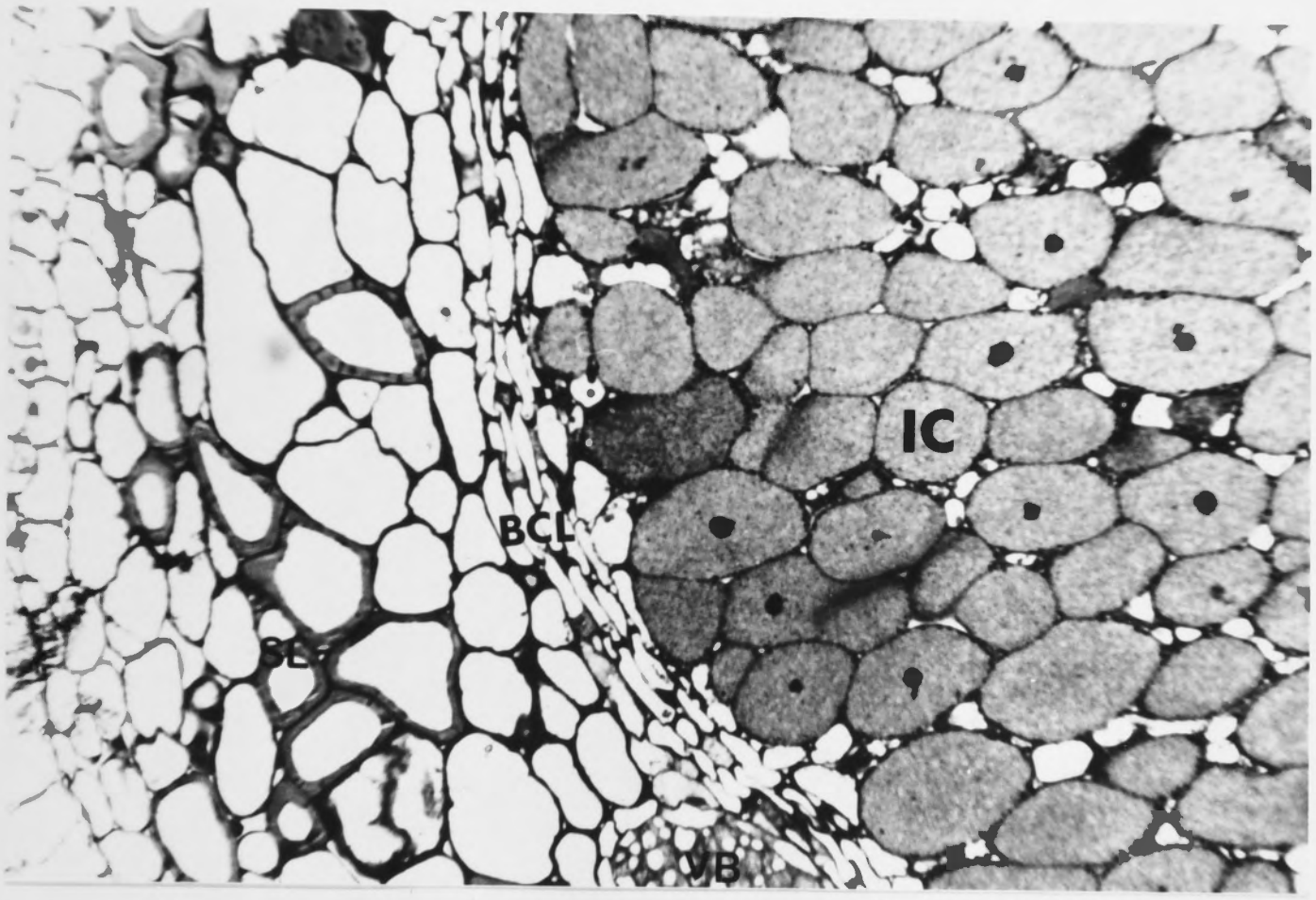
4.3.3 Late nodule development

Figure 4.2 presents a light microscope section through the nodules of nod49 (a) and Bragg (b). Nodules formed on the mutants nod49, nod139, nod772 and *rj*$_1$(Lee) were normal in development and similar to Bragg nodules when observed by light microscopy. Furthermore, the results in Table 4.1 indicate that the nodules on the non-nodulating mutants can be effective in acetylene reduction. Often these occasional nodules were very large as shown in Figure 4.3a for nod49. For example, the mean fresh weight of nodules on nod49 which were formed when inoculated with *B. japonicum* strain CB1795 was 113 mg compared to 11.2 mg for Bragg. Such results are commonly found in plants which have a low number of nodules. In these cases, it seems that the plant compensates for its low number of nodules by producing nodules of higher mass so as to maintain a constant mass of nodule tissue. Occasional nodules on the non-nodulating mutants were sometimes concentrated at the cotton plug region of the Leonard jar as indicated in Figure 4.3b for nod49. Generally, nodules on nod49, nod139, nod772 and *rj*$_1$(Lee) were located on the root tissue that developed much later during plant growth and rarely on the upper portion of the tap root. On the other hand, nodules on Bragg were mainly seen at the upper portion of the tap root. Nodules on nts382 were located on both the tap and lateral root at a higher density in a beaded manner.

Figure 4.2 **Light microscope section through the nodules of nod49 (a) and Bragg (b).** Note that nod49 is similar to Bragg in both nodule development and anatomy. The plants were grown in Leonard jars with *B. japonicum* strain CB1795 at 10^8 viable cells. ml^{-1} and harvested 4 weeks after planting.

IC - infected cells
BCL- boundary cell layer
VB - vascular bundles
SL - scleroid layer

(a)



(b)

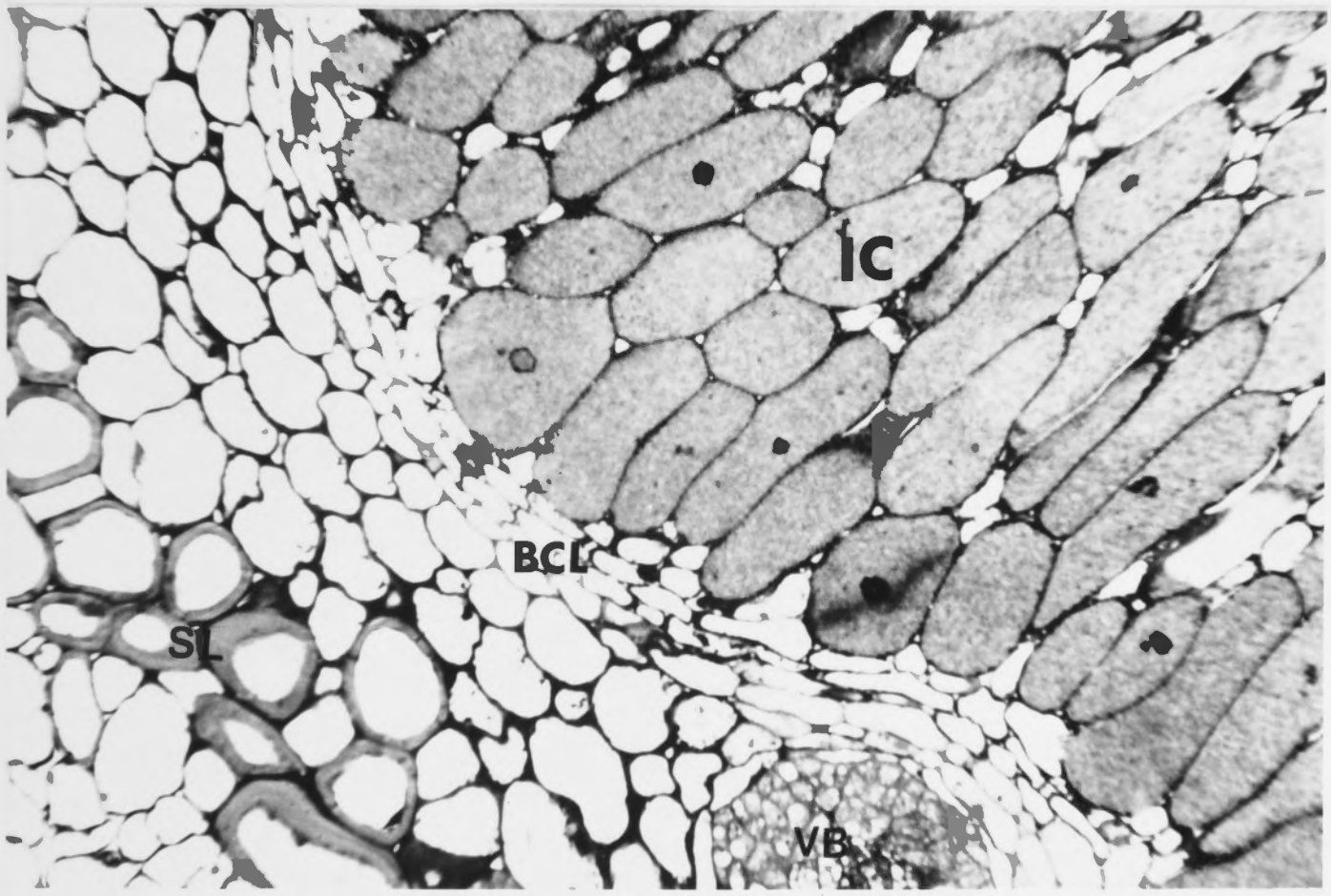
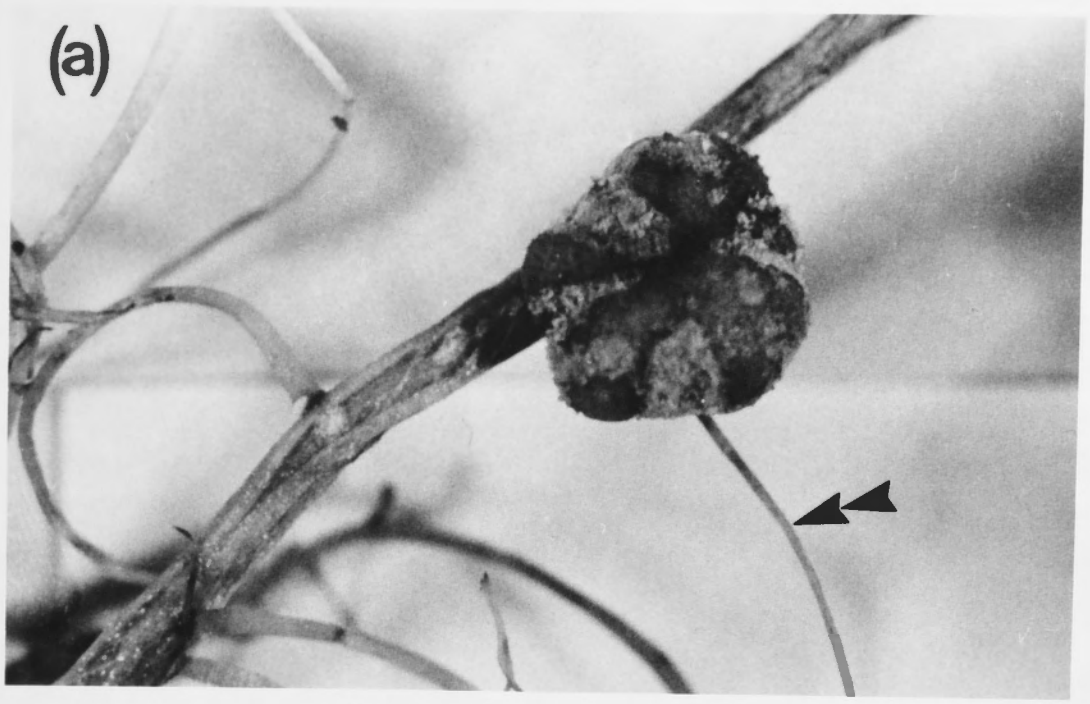
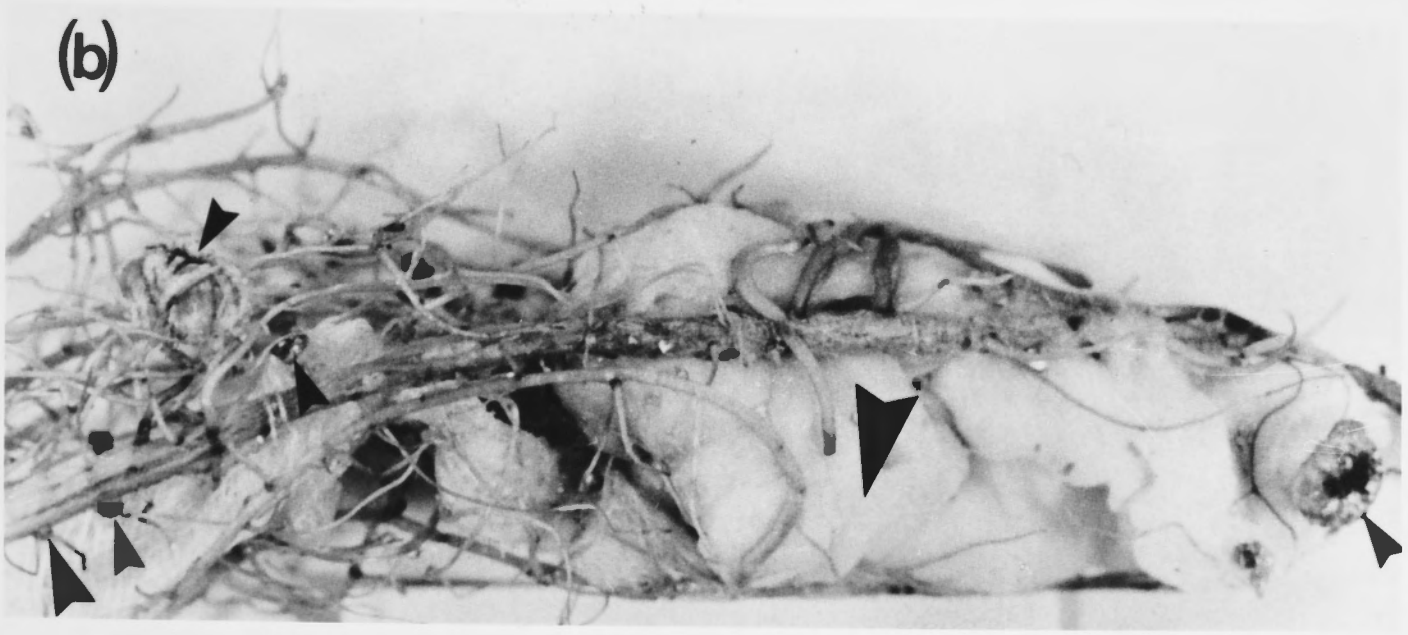


Figure 4.3 **(a) Occasional nodule on the non-nodulation mutant nod49.** Note that the nodule is associated with the emergence site of a lateral root indicated by the double arrow. Plants were grown in Leonard jars and inoculated with *B. japonicum* strain CB1795 at 1 x 10^9 viable cells. ml^{-1}.

(b) Nodules on nod49 at the cotton plug region of the Leonard jar. Plants were grown as above. The large arrow indicates the cotton plug. The smaller arrows indicate the nodules at the cotton plug.


(a)


(b)

4.3.4 Attempts to suppress the non-nodulation phenotype

This section deals with results of a range of experiments designed in an attempt to suppress the non-nodulation phenotype.

4.3.4.1 High dose of *Bradyrhizobium* and *Rhizobium* strains

Twenty three *Bradyrhizobium* and *Rhizobium* strains that normally nodulate Bragg failed to nodulate nod49 at low inoculant titres. However, at high dose inoculation some strains were successful in forming a few nodules on the mutants (Table 4.1). *B. japonicum* strains CB1809, USDA110 and USDA123 consistently nodulated the parent cv. Bragg and supernodulated nts382. As described in Section 4.3.1, occasional nodules were formed on the non-nodulation mutants. Mutant nod772 which was leaky for root hair curling (Table 4.2) but allelic to rj_1 (Chapter 3) had a higher number of nodules compared to the other non-nodulating mutants of Bragg (Table 4.1). Strain USDA76 which was capable of some nodule induction in rj_1 plants was also capable of nodule initiation in the induced non-nodulation mutants of soybean. Nodule-like structures, defined as swellings of the root, were noted in several instances with different bacterial strains. Anatomical analyses of these showed extensive multiplication of cells without any bacteroid tissue.

Rhizobia were isolated from nodules which formed occasionally on nod49 and retested for nodulation on this mutant. The reisolated rhizobia failed to nodulate nod49 to any greater extent indicating that these isolates were not genetic variants of the parent strain with altered nodulation capabilities.

4.3.4.2 Combinations of *Bradyrhizobium* and *Rhizobium* strains

To study the effect of the combination of *Bradyrhizobium japonicum* strains, bacteria were used in combination to form inoculation mixtures and were used as multi-strain inoculants. *B. japonicum* strains USDA110 and CB1809 when used in combination resulted in an average of 2 nodules. plant^{-1} on nod49 and 36

nodules. plant^{-1} on Bragg. The combination of 23 strains of rhizobia resulted in an average of 2 nodules. plant^{-1} on nod49 and 16 nodules. plant^{-1} on Bragg using medium inoculant numbers (10^7-10^8 viable cells. plant^{-1}). Thus the use of multi-strain combinations of rhizobia did not improve nodulation to any greater extent.

4.3.4.3 The effect of cotyledon removal and addition of nitrate on nodulation

The translocation of an inhibitor of cortical cell divisions in pea from the cotyledons to the developing root through the phloem was suggested by Phillips (1971b). Phytohormones such as abscisic acid (ABA) have been shown to inhibit nodule formation in peas and were postulated to act by the reduction of cytokinin-stimulated cortical cell mitoses (Phillips, 1971a). In an attempt to remove such sources of potentially inhibitory substances, cotyledons were detached from seedlings at one week after germination. Furthermore, it was tested whether the non-nodulation mutants were symbiotically deficient as a result of delayed nodule initiation. This could then lead the seedlings into a period of severe nitrogen starvation. Such stressed plants may be incapable of further symbiotic development. Accordingly, 0.5 mM KNO_3 was added to the nutrient solution to maintain relatively fit seedlings without affecting the nodulation potential of the plants.

Table 4.3 shows the effect of the removal of cotyledons one week after germination and the effect of 0.5 mM KNO_3 on nodulation. All plants were inoculated with the strain CB1809 and cultured in Leonard jars. Uninoculated plants served as controls. It was observed that neither cotyledon removal nor nitrate supplementation allowed the non-nodulating mutants to nodulate. The removal of cotyledons resulted in high nodule number in Bragg and nts382 but a decrease in nodule dry weight and plant weight. This can be explained by the loss of cotyledonary reserves needed in early plant growth. The addition of 0.5 mM KNO_3 both increased nodule number in Bragg and nts382 and increased plant dry weight in all the genotypes. Non-nodulation mutants had higher plant

Table 4.3 **Effects of cotyledon removal and addition of 0.5 mM KNO_3 on nodulation of Bragg, nod49, nod139, rj_1(Lee) and nts382.** Plants were grown in Leonard jars and inoculated with 1 ml of *B. japonicum* strain CB1809(=USDA136) at 1 x 10^8 viable cells. ml^{-1}. Cotyledons were excised a week after germination. 0.5 mM KNO_3 was added to the plant nutrient solution in the Leonard jar. Data are means of 2 to 5 plants.

Table 4.3

Nodulation parameters	Treatment: Uninoculated	CB1809 (=USDA136)	CB1809 minus cotyledon	CB1809 plus 0.5 mM KNO_3
		Bragg		
Nodule No.	0	30	40	54
Nodule dry wt. (mg)	0	59	46	73
Plant dry wt.(mg)	1748	1061	670	1105
		nod49		
Nodule No.	0	0	0	0
Nodule dry wt. (mg)	0	0	0	0
Plant dry wt.(mg)	1024	953	554	1799
		nod139		
Nodule No.	0	0	0	0
Nodule dry wt. (mg)	0	0	0	0
Plant dry wt. (mg)	1056	899	959	1335
		***rj*$_1$(Lee)**		
Nodule No.	0	0	0	0
Nodule dry wt. (mg)	0	0	0	0
Plant dry wt. (mg)	1167	896	754	1553
		nts382		
Nodule No.	0	419	428	431
Nodule dry wt. (mg)	0	102	93	120
Plant dry wt.(mg)	1043	541	463	700

dry weights when grown with 0.5 mM KNO_3. In another experiment, the mutants were also given a high amount of nitrate (7.5 mM KNO_3) thrice a week for 2 weeks, then completely deprived of nitrate and later inoculated with a high dose of *B. japonicum* USDA110. This was done to observe whether nitrogen starvation may enable them to nodulate and fix nitrogen. No nodulation was observed on all the non-nodulating mutants, whereas Bragg had 261 ± 134 nodules. $plant^{-1}$ and nts382 had 987 ± 257 nodules .$plant^{-1}$ (Table 4.4).

4.3.4.4 Vegetative propagation of soybean mutants

The delayed nodulation observed in the non-nodulation mutants may represent a developmental restriction. In other words, during early seedling development these mutants may be resistant to *Bradyrhizobium* . To test this hypothesis, plants were vegetatively propagated via cuttings to obtain ontogenetically maturer plants with young roots. Two week old seedlings were excised and vegetatively propagated by cuttings. The cut was made just above the cotyledon and rooted in a potting mixture. The rooted cuttings were transferred to large pots containing sand and vermiculite and cultured in the greenhouse for five weeks. Again, no nodulation was observed on the non-nodulation mutants as shown by the results in Table 4.5, indicating that the phenotype is not ontogenetically restricted.

4.3.5 Susceptibility to *Phytophthora megasperma* F. sp. *glycinea* Rot

Mutant nod772 was found to be susceptible to *Phytophthora* rot when grown in a 2 : 1 sand-vermiculite mixture and after the pots had remained wet for several days. However, Bragg and mutants nod49, nod139, rj_1(Lee) and nts382 were not susceptible to the disease. The seed rot and preemergence damping-off form of the disease were sometimes observed. Often symptoms were typical of late infected plants, that is, yellowing of lower leaves followed by

Table 4.4 **Effect of high nitrate on the nodulation of Bragg and its mutants.** Plants were grown in a 2 : 1 mixture of sand and vermiculite, watered with 7.5 mM KNO_3 thrice a week for 2 weeks, starved of nitrate and later inoculated with a high dose (1×10^9 viable cells. ml^{-1}) of *B. japonicum* strain USDA110. Each entry in the table represents the mean of 9 plants ± S.D.

Table 4.4

Genotype	Nodule no.	Plant dry wt. (g)	nmol. C_2H_4. $pl^{-1}.h^{-1}$
Bragg	261 ± 134	2.6 ± 0.4	80 ± 5
nod49	0	2.0 ± 0.5	0
nod772	0	2.1 ± 0.5	0
nod139	0	3.0 ± 0.5	0
rj1(Lee)	0	3.5 ± 0.6	0
nts382	987 ± 257	1.8 ± 0.4	123 ± 19

Table 4.5 **Vegetative propagation of soybean mutants and the wild-type Bragg.** One week old seedlings were vegetatively propagated by cutting them above the cotyledons and rooting them in a potting mix. Cuttings were inoculated with *B. japonicum* strain USDA110 at 1×10^8 viable cells. ml^{-1}. Data are means of 2 to 5 plants.

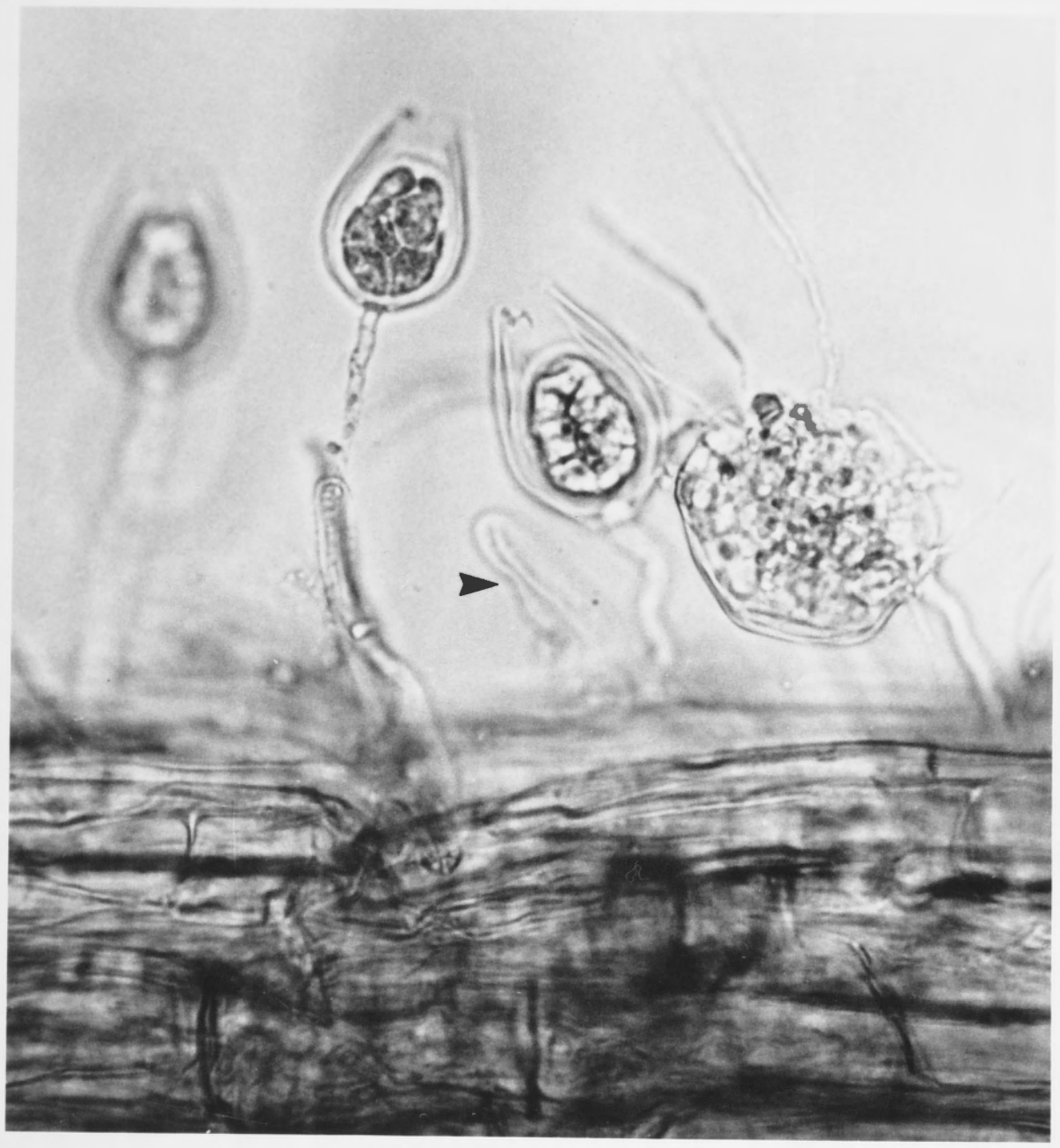
Table 4.5

Plant genotype	Nodule no. plant^{-1}	Nodule dry weight. plant^{-1}(g)	Shoot dry weight. plant^{-1}(g)	Root dry[a] weight. plant^{-1}(g)
Bragg	110	0.181	4.368	1.675
nod49	0	0	2.825	1.309
nod772	0	0	3.314	1.738
nod139	0	0	2.976	2.134
rj$_1$(Lee)	0	0	4.164	3.156
nts382	716	0.350	1.350	0.666

[a] root dry weight includes nodule dry weight

the older leaves becoming chlorotic with complete wilting of the plant. The infected roots when examined were dark brown and had discoloured cortex and vascular tissue. Normally large plants wilted and died very quickly. Microbiological examination of the roots revealed the occurrence of sporangiophores which were simple and indeterminate. Obpyriform terminal sporangia (conidia) were observed as depicted in Figure 4.4. Germinating sporangia extruded zoospores into a thin, delicate membranous vesicle. This diagnosis was also confirmed by the Crop Disease Diagnostic Service of the New South Wales Department of Agriculture when *Phytophthora megasperma* F. sp. *glycinea* was isolated from affected stems of nod772. The susceptibility to the disease in nod772 segregated into diseased and normal at the ratio of 3 : 1 in the F_2 progeny in nod772 backcrossed to the wild-type Bragg.

Figure 4.4 **Obpyriform terminal sporangia (conidia) of *Phytophthora megasperma* F. sp. *glycinea*** observed on diseased roots of nod772. The arrow indicates a root hair on the nod772 root.



4.4 DISCUSSION

The non-nodulation soybean mutants and the supernodulation mutant nts382 (Carroll *et al.*, 1985a,b; 1986) were characterized and the stage(s) of alteration of nodulation in these mutants were studied. Comparisions were made to the naturally-occurring non-nodulation mutant *rj*$_1$ isolated by Williams and Lynch (1954). Compared to Bragg mutant nts382 had a similar proportion of curled root hairs a week after inoculation with *B. japonicum* strain USDA110 and a slightly increased number of curled root hairs two weeks after the inoculation. All the non-nodulation mutants were affected in root hair curling. Mutants *rj*$_1$(Lee), nod49 and nod139 did not have curled root hairs after inoculation with *Bradyrhizobium japonicum* strain USDA110. Mutant nod772, on the other hand, was leaky and had a few curled root hairs and formed a few more nodules compared to the other mutants. Thus, the non-nodulation mutants are blocked at a very early stage of nodulation either at or prior to root hair curling. The main steps that occur prior to or concomitant with hair curling are colonization, attachment and cortical cell divisions. Details of these steps have been investigated and reported in Chapter 5.

Nodules when formed on the non-nodulation mutants with the exception of *rj*$_1$ were always located on the root tissue that developed much later in plant growth indicating that nodulation was both delayed and suppressed. However, the development of such nodules was morphologically normal and similar to wild-type Bragg nodules. Nodules on these mutants also fixed nitrogen. This evidence further indicated that the blockage in nodulation was confined to an early stage. Sometimes, the nodules were very large and often occurred at the cotton plug region of the Leonard jar. This was the region where the narrow neck of the vermiculite compartment dipped into the reservoir compartment. This may be caused by an increased concentration of rhizobia in this region after inoculation. Separate experiments showed that high numbers of *Bradyrhizobium* were needed for any nodulation of the mutants. Alternatively, certain bacterial by-product(s) and other compounds which enhance nodulation could accumulate at this region of the Leonard jar. Nodulation occurred with some *B. japonicum*

strains at high inoculant titres (above 10^8-10^9 cells. plant^{-1}). Therefore, high cell numbers of rhizobia may be necessary to provide sufficient cells capable of infection (La Favre and Eaglesham, 1984) possibly by the increased synthesis of bacterial signals. The accumulation of a bacterial signal (as would be required for the induction of cortical cell divisions) at the cotton plug of the Leonard jar may explain why nodulation was often seen at this region. A similar situation may also occur in different sand/soil mixtures with an organic substrate increasing both bacterial numbers and possible production or retention of signal substances.

In an attempt to exclude any inhibitory factors present in the cotyledons which might inhibit nodulation, cotyledons were removed one week after germination. This did not enhance nodulation in the non-nodulation mutants and indicated that the cotyledons are not involved in the non-nodulation response. The possibility that the non-nodulation mutants may be slow to nodulate and nodulation (when it occurs) may occur on the root tissue which developed later in plant growth was tested by vegetatively propagating the seedlings. The experiment was designed to exclude any inhibitors in the cotyledons that may prevent nodulation. However, these cuttings failed to nodulate. Small amounts of nitrate are known to help in nodulation (Carroll *et al.*, 1985b). Supplementing the nutrient solution in the Leonard jars with 0.5 mM KNO_3, which was not inhibitory to nodulation and nitrogen fixation at this concentration for the wild type, did not help the non-nodulation mutants to nodulate. Likewise, the addition of a high amount of nitrate, followed by a period of nitrate deprivation and subsequent inoculation with *B. japonicum* USDA110 did not help to overcome the non-nodulation problem.

The non-nodulating mutants of soybean are considered to be resistant to nodulation. However, they form a few nodules in Leonard jars when inoculated with a high cell number of rhizobia. In spite of this they are referred to as non-nodulating mutants, as they do not form nodules at low inoculant cell numbers and do not nodulate in the field or in sand - vermiculite mixtures. Clark (1957) observed that the mutation rj_1 (Williams and Lynch, 1954) did not nodulate in three different soils. Mutant nod772 appears to be a leaky mutant,

yet it is allelic to *rj*$_1$ (Chapter 3). It consistently formed a higher number of nodules compared to the other non-nodulation mutants and had a smaller proportion of markedly curled root hairs when inoculated with a medium inoculant cell number of *B. japonicum* strain USDA110. It was also susceptible to *Phytophthora megasperma* F. sp. *glycine* rot at both the seedling and the adult plant stages. It is unlikely that this feature is related to the non-nodulation mutation since neither nod49 nor *rj*$_1$(Lee) were susceptible to this fungal pathogen.

Mutants nod49, nod139 and nod772 are the first induced genetic variants for non-nodulation in soybeans. Even though Williams and Lynch isolated the naturally-occurring non-nodulation (*rj*$_1$) mutant in 1954 and non-nodulation mutants have been reported in other legume species since then, an extensive study of the nature of the non-nodulation phenomenon and the exact blockage steps in symbiotic development has not been done. The non-nodulation mutants that have been isolated can be used as an excellent tool in the elucidation of the mechanisms and functions involved in nitrogen fixation. The further characterization of the other aspects of these mutants is the subject of the subsequent chapters.

CHAPTER 5

DEVELOPMENTAL STAGE OF ALTERATION IN NODULATION IN SOYBEAN MUTANTS

5.1 INTRODUCTION

The contribution of the legume to the understanding of the mechanisms and functions of the various developmental stages of nodulation can be understood by fragmenting and characterizing the different steps of this process. Several mutants with varying nodule numbers have been identified in soybean (Williams and Lynch, 1954; Carroll *et al.,* 1985a,b; 1986; Mathews, *et al.,* 1987b), but the precise alteration in the early ontogeny of nodule formation has not been described.

The process of cellular recognition involving the legume and the rhizobia was believed to arise from a specific union between chemical receptors on the surface of interacting cells (Burnet, 1971) and the involvement of host lectins in attachment and infectivity had been reported by Stacey *et al.* (1980), Dazzo *et al.* (1984) and Seegers and LaRue (1985). In addition, components of legume root exudates such as flavones (Peters *et al.,* 1986; Redmond *et al.,* 1986), isoflavanones (Firmin *et al.,* 1986) and isoflavones such as daidzein and genistein (Kosslak *et al.,* 1987) are known to induce *nod* gene expression in *R. meliloti, R. trifolii, R. leguminosarum* and *B. japonicum,* respectively. Excretion of a nodulation-inhibitory substance by the naturally-occurring non-nodulation variant rj_1 when grown in co-culture with the near isogenic (Rj_1) line was reported by Elkan (1961) but Eskew and Schrader (1977) were unable to confirm this observation.

Growth and multiplication of rhizobia in the rhizosphere are important in nodule initiation. Elkan (1962) observed no inhibition in the growth of bradyrhizobia in the rhizosphere of the rj_1 plants. A higher number of total bacteria (about 20 per cent) on the roots of the nodulating plant compared to the non-nodulating rj_1 plants was reported by Clark (1957). Payne and Pueppke (1985) observed that the roots of the rj_1 plants also were not defective in their ability to bind bradyrhizobia. The precise stage of blockage of nodulation in rj_1

plants had not been reported earlier. In chapter 4 and Mathews *et al.* (1987a), a defect of the *rj*$_1$(Lee) plants at the root hair curling stage was described.

The region of the root just below the position of the smallest emerging root hair is the infectible region of the wild-type soybean and cowpea roots, that is, between the RT and SERH zone (Bhuvaneswari *et al.,* 1980; 1981) as illustrated in Figure 1.1. Nodulation efficiency or the position of nodule formation relative to this zone can be used as an indicator of the rapidity of the responses leading to infection and nodulation. It has been reported that division and proliferation of the cortical cells start ahead of the infection thread (Libbenga and Harkes, 1973). In soybean (*Glycine max* L.), Bauer *et al.* (1985) observed initiation of cell divisions in the cortex of the roots when *B. japonicum* cells were placed in contact with the roots but separated by millipore membranes. The nature of the bacterial signal(s) involved in sub-epidermal cell divisions is not known but auxins and cytokinins of bacterial origin may have a regulatory role in those cell divisions and nodule initiation (Phillips and Torrey, 1970; Libbenga *et al.,* 1973).

Sub-epidermal cell divisions not associated with infection threads were observed by Calvert *et al.* (1984) and were called pseudoinfections. These divisions were usually restricted to a few cells of both the hypodermis and a few of the outer cortical cell layers. Pseudoinfections were commonly found in the developing and younger regions of the root and often without an associated root hair (Calvert *et al.,* 1984). In soybean, root hair curling was observed 6 h after inoculation and infection thread formation was observed at 18 h after inoculation (Turgeon and Bauer, 1982; 1985). Calvert *et al.* (1984) observed that the first hypodermal cell division was complete by 12 h after inoculation, indicating that host cell divisions by the bacteria occur soon after inoculation and prior to infection thread formation. Furthermore, the presence of sub-epidermal cell divisions (scd) without root hair curling suggested that scd may be independent of both root hair emergence and induced root hair deformation (Calvert *et al.,* 1984). Truchet *et al.*(1984) and Hirsch *et al.* (1984) obtained similar results while examining pseudonodules on alfalfa induced by *Agrobacterium tumefaciens* and *Escherichia coli* containing nodulation genes from the *R. meliloti* symbiotic plasmid. They observed that transconjugants were able to stimulate

nodule meristem formation without root hair infection thread formation and sometimes without root hair deformation. It was found that the majority of infections stopped developing at relatively early stages of cell division (stages I - V) in the soybean cv. Williams (Calvert *et al.,* 1984) and at stage II in cv. Clark (Pueppke and Payne, 1987), such that most infections do not result in nodule formation.

This chapter details the studies carried out to determine the precise site of alteration in nodule initiation in the supernodulation and non-nodulation mutants of the soybean cv.Bragg. Observations were made on the preinfection events such as rhizosphere colonization, *Bradyrhizobium* attachment, sub-epidermal cell divisions and infection events. The implications of this chapter to the understanding of the nodulation process is also discussed.

5.2 MATERIALS AND METHODS

5.2.1 Plant material and *Bradyrhizobium* strains

Soybean cv. Bragg and its non-nodulating mutants (nod49, nod772 and nod139), the supernodulating mutant (nts382), the intermediate supernodulating mutant (nts1116) and the spontaneous mutation rj_1 (Lee) were used in these experiments.

The co-culture and effect of preincubation of *B. japonicum* in the root exudate on nodulation experiments were conducted using *Bradyrhizobium japonicum* strain USDA110. Strain USDA1-110ARS (azide, rifampicin and streptomycin resistant) used in the root attachment studies was obtained from Dr. W.D. Bauer (Ohio, USA). Strain CB1809 was used for the rhizosphere colonization experiment. The early infection events in these soybean mutants were studied by inoculating the roots with *Bradyrhizobium japonicum* strains CB1795 and USDA110. Strains CB1809 and CB1795 were obtained from Mr. John Brockwell of C.S.I.R.O., Canberra, Australia.

5.2.2 Co-culture of the non-nodulating mutants with the supernodulation mutant or the wild-type cv. Bragg

The co-culture studies were conducted in modified Leonard jars. Six germinated seedlings of different genotypes were grown in each Leonard jar. Mutants nod49, nod772, nod139 and rj_1 were co-cultured with either nts382 or Bragg at the rate of 3 per genotype. The seeds were inoculated with a peat culture of *B. japonicum* strain USDA110 containing 10^8 cells. g^{-1} at planting and were cultured in a naturally-lit glasshouse (16 -28^oC) or in a growth

chamber (16 h day, 28°C; 8 h night, 30°C, 380 μ einsteins. $m^{-2}.s^{-1}$light intensity). Observations were taken for nodulation and plant growth at 4 weeks after germination. Co-culture in pots containing a 2 : 1 sand-vermiculite mixture was studied by growing 20-25 seedlings of each non-nodulating mutant in close proximity with the same number of seedlings of either the supernodulation mutant nts382 or the wild-type Bragg. Plants were inoculated at planting with a peat culture of *Bradyrhizobium japonicum* strain USDA110 and cultured on nitrogen free nutrients. Observations were taken 4 weeks after germination.

5.2.3 Preincubation of *Bradyrhizobium japonicum* USDA110 prior to inoculation of wild-type soybean

The effect of mutant root exudate on nodulation of the wild-type Bragg was examined to determine whether the non-nodulation mutants differed in the root exudates from both the supernodulation mutant and the wild type. Also, whether incubation of *Bradyrhizobium japonicum* strain USDA110 in the exudate of the mutants could alter the nodulation efficiency of the wild-type Bragg. Sterile plastic growth pouches (obtained from Scientific Products, Evanston, Illinois) were used for the nodulation tests on Bragg. Germinated Bragg seeds were transferred aseptically into pouches, which had been previously watered with half strength Jensen's plant nutrient solution, at the rate of two per pouch. Plants were cultured as described by Bhuvaneswari *et al.* (1980) and were maintained in a Conviron growth chamber set at 26°C night and 28°C day temperatures. The relative humidity was maintained at 85 per cent with 16 h photoperiod. The pouches were wrapped in aluminium foil, placed upright in racks and watered to saturation. Extreme care was taken to maintain the optimum quantity of nutrient solution in the pouches (just sufficient to wet the filter paper) and to avoid water logging conditions. *Bradyrhizobium japonicum* strain USDA110 was preincubated in the exudate of the mutants. The exudate of either the wild type or the mutants was collected, as described by Halverson and Stacey (1985), with the exception that a 100 ml crystallization dish covered by a Petri plate top containing eight 0.5 cm diameter holes was used for collecting the root exudates.

Two day old germinated seedlings were inserted into the sterilized nutrient solution through the holes on the Petri plate with the root system touching the plant nutrient solution. The root exudate was collected after 10 days of culture.

The position of the root tip (RT) and the smallest emerging root hair (SERH) of the wild-type test plants were marked on the pouch surface using a stereoscope. The plants were cultured as described above and inoculated with 1 ml. plant^{-1} of the *Bradyrhizobium japonicum* strain USDA110 preincubated in the exudate. Plants were scored for nodulation between the RT-SERH zone. Nodulation above the root tip mark was used to indicate the efficiency of nodulation since bradyrhizobia nodulating below the RT mark are considered to be delayed in nodulation.

5.2.4 Electrophoretic analysis of root protein

For the protein analysis of the root, plants were grown in a 2 : 1 sand-vermiculite mixture and either inoculated or uninoculated. The uninoculated plants were cultured in sterilized sand-vermiculite. Roots of 5 day old seedlings of all the genotypes grown in the presence and absence of *Bradyrhizobium japonicum* strain USDA110 were used to study the protein pattern. Root proteins were extracted by grinding the roots under liquid nitrogen with insoluble polyvinyl pyrolidone (PVP). The homogenate was then extracted with DL-dithiothreitol (DTT), filtered through a Whatman No. 1 filter paper under vacuum followed by heat extraction at 100^{o}C in a water bath. The crude protein was spun at 31,000 g for 10 min. The protein was precipated using acetone, cooled and respun to concentrate it. It was then dried under a nitrogen stream and stored in a -20^{o}C freezer. The protein was then dialysed for 16 - 20 h at 4^{o}C against degassed distilled water. It was then spun in an Eppendorf centrifuge for 5 min, dried under a nitrogen stream and stored in a -20^{o}C freezer. The protein sample was prepared for gel electrophoresis by dissolving the pelleted protein in a sample buffer containing DTT. It was vortexed vigorously, then sonicated and heated to dissolve completely. It was then spun at 12,000 g in an Eppendorf centrifuge for 5 min and the supernatant was loaded onto a one

dimensional (15 per cent acrylamide) SDS-PAGE gel (Laemmli system) and run through the stacking gel with a 25 mA current and the separating gel with a 35 mA current (A. Krotzky, *pers. comm.*). The gel was then stained with silver stain as described by Merril *et al.* (1979) and modified by A. Krotzky (*pers.comm.*).

5.2.5 Rhizosphere colonization

The soybean mutants were grown in a vertisol (collected near Breeza, New South Wales, Australia) which was rich in organic nitrogen and free of *Bradyrhizobium japonicum* . Black plastic pots, 25 cm in diameter, were packed with 5 kg soil mixed with an equal volume of well-washed vermiculite which was inert, N- free and contained no rhizobia. Each pot was sown with 12 soybean seeds. Each seed was inoculated with one ml of a suspension of a pure commercial peat culture of *B. japonicum* in a seedling salts solution (Cannon *et al.*, 1967). Three inoculation treatments used in the experiment were: n (normal, rate recommended by inoculant manufacturer) - 2.87×10^5 viable cells. ml^{-1}; n/100 - 2.87×10^3 viable cells. ml^{-1}; 100n - 2.87×10^7 viable cells. ml^{-1}. For all the genotypes, three replicate pots of each inoculation treatment were set up. Pots were arranged at random in a glasshouse controlled at 23°C (day, 10 h) and 18°C (night, 14 h) Plants were sampled 7, 12 and 17 days after emergence to determine the number of *B. japonicum* in the rhizosphere.

Glycine soja (Sieb. and Zucc.) synonymous to *Glycine ussuriensis* (Regel and Maack) was used as the test plant to enumerate the population of *Bradyrhizobium japonicum* in the rhizosphere (Brockwell *et al.*, 1975). Seeds of *G. soja* were scarified using concentrated H_2SO_4 for 20 minutes and washed in about 10 changes of sterile distilled water to remove all traces of acid. The seeds commenced to imbibe during washings. Sterilized seeds were allowed to soak in sterile distilled water overnight in the refrigerator. Imbibed seeds were spread on water agar (2 per cent w/v) in Petri plates and placed overnight in a refrigerator (4°C). The seeds were then moved to a 25°C incubator and incubated in an

inverted position for 1 day to obtain seedlings with straight roots. Seedlings with 1 - 2 cm long roots were transferred aseptically with fine forceps into plant infection tubes (15 x 2.5 cm) containing 12 ml of a soft seedling agar minus nitrogen (Cannon *et al.*, 1967). The composition of this seedling agar is detailed in Table 5.1.

A ten-fold serial dilution plant infection test was employed to enumerate populations of *B. japonicum* in the rhizosphere (Brockwell *et al.*, 1975) of the soybean lines. At every sampling, each seedling was carefully uprooted and the root system suspended in 100 ml of seedling solution in a plastic bag. The rhizosphere contents were dispersed into the suspension using a stomacher (Sharpe and Jackson, 1972) operating at approximately 100 cycles. min^{-1}. Using a 1 ml pipette with a wide tip for rapid delivery, 1 ml of the suspension was transferred into 9 ml of the seedling solution. A fresh pipette was used at each dilution. Three 1 ml portions were used to inoculate three test plants and a fourth millilitre was used to make the next ten-fold dilution in a similar manner. The series was repeated 6 times, thus using 18 test plants. The test plants were grown for 6 weeks after inoculation, then observed for nodulation. The most probable number of *Bradyrhizobium* in the rhizosphere was calculated from the proportion of plants forming nodules at each dilution level using the modified version of Fisher and Yates (1963) tables outlined in Brockwell *et al.* (1975).

5.2.6 *Bradyrhizobium* attachment assay

Bradyrhizobium japonicum strain USDA1-110ARS was grown in yeast extract mannitol-gluconate broth (Bhuvaneswari *et al.*, 1980) to early logarithmic phase. Dilutions of the culture were made with sterile distilled water to obtain approximately 10^4 cells. ml^{-1}. Surface sterilized seeds (Chapter 4) were placed on Petri plates containing one per cent water agar and incubated at 28°C for 48 h. Seedlings with straight roots 3 - 4 cm long were selected and cut between 4 mm and 14 mm above the root tip. Sets of 10 root segments were added to 80 x 100 mm Pyrex culture dishes containing 30 ml of bacterial suspension. The root segments were incubated for 15 min on a rotary shaker at

Table 5.1 **Composition of seedling nutrient agar.** The medium was prepared by melting the agar in distilled water and then adding the nutrients. This was dispensed into plant infection tubes and autoclaved for 20 mins at 15 p.s.i.

Table 5.1

	Ingredients	litre^{-1}
	Agar	7.5 g
	Distilled water	958.0 ml
	Solution A	10.0 ml
	Solution B	20.0 ml
	Solution C	10.0 ml
	Solution D	1.0 ml
	Solution A-Z	1.0 ml
Solution A		
	K_2HPO_4	50.0g
	KH_2PO_4	50.0g
Solution B		
	$Ca_3(PO_4)_2$	100.0g
	$FePO_4$	25.0g
	$CaCO_3$	5.0g
Solution C		
	$MgSO_4$	20.0g
	NaCl	10.0g
Solution D		
	$FeCl_3$ solution	16.8 ml
	EDTA salt	2.0 g
Solution A-Z		
	H_3BO_3	2.860g
	$MnSO_4.4H_2O$	2.030g
	$ZnSO_4.7H_2O$	0.222g
	$CuSO_4.5H_2O$	0.079g
	$H_2MoO_4.H_2O$	0.090g

50 rpm. The loosely attached bacteria on the roots were removed by vigorously shaking the root segments in 700 ml of Hoagland's solution. The composition of this solution is outlined in Table 5.2. The root segments were then sonicated to detach the firmly attached bradyrhizobia and the bacterial suspension was plated on a spiral plater Model DUL Plater (Cincinnati, Ohio) to obtain the cell numbers as described by Vesper and Bauer (1985).

In experiments testing the effect of preincubation of *B. japonicum* strain USDA1-110ARS in various root exudates on attachment, three day old seedlings of the soybean mutants were used. The seedlings were inserted into test tubes containing 2 ml sterile half strength Jensen's plant nutrient solution and inclined at 45^{o} with their root tips touching the surface of the solution. The root exudate was collected for 24 h and filter sterilized using a 0.45µm filter. 50 ml of the mid-logarithmic phase cultures of USDA1-110ARS (about 10^{9} viable cells. ml^{-1}) was concentrated by centrifugation at 7,000 g for 10 mins washed once in 20 ml Jensen's plant nutrient solution and suspended into 10 ml of the exudate. Cell numbers of USDA1-110ARS were determined at this stage and after incubation for 18 h in the root exudate using the spiral plater. Jensen's plant nutrient solution was used as a control in these experiments.

5.3.7 Early infection studies

Germinated seedlings were transferred to plastic growth pouches and cultured as described earlier (Section 5.2.3). The position of the RT-SERH zone was marked on the pouch surface. *Bradyrhizobium japonicum* strain CB1795 and USDA110 were grown to mid logarithmic phase in yeast extract mannitol broth and diluted with sterile water to give 1 x 10^{5} and 1 x 10^{9} viable cells. ml^{-1} dilutions which were determined by plate counts. Plants were inoculated with these dilutions at the rate of 1 ml. root $^{-1}$ and harvested 5 days after inoculation. Two root segments of 7 to 8 cm length were examined for roots of all the genotypes inoculated with *B. japonicum* strain CB1795 at both the cell titres (1 x 10^{5} viable cells. ml^{-1} and 1 x 10^{9} viable cells. ml^{-1}) while one root segment of the same size was examined for all the genotypes inoculated with *B. japonicum* strain USDA110. Prior to removing the roots from the pouches,

Table 5.2 The composition of Hoagland's nutrient solution.

Table 5.2

Chemical	Litre^{-1}
$MgSO_4.7H_2O$	0.49g
$CaCl_2.2H_2O$	0.10g
KH_2PO_4	0.02g
K_2HPO_4	0.14g
Trace elements[a]	1 ml
Sequestrene 2.35 g. l^{-1}	2.35mg
K_2SO_4	0.25g

[a] **Trace element stock (Salisbury and Ross, 1978)**

Chemical	g. litre^{-1}
$MnCl_2.4H_2O$	0.5 Mn; 0.65 Cl
H_3BO_3	0.5 B
$ZnSO_4.7H_2O$	0.05 Zn
$CuSO_4.7H_2O$	0.02 Cu
$H_2MoO_4.H_2O$	0.05 Mo

partial cuts were made at the RT and SERH marks to enable the identification of the reference points in the root sections. A 70 to 80 mm root segment was removed from each root comprising 30 mm above the SERH and 30 to 40 mm below the RT mark made at the time of inoculation. The root segments were fixed overnight in 3 per cent glutaraldehyde buffered with 50 mM phosphate to pH 7.2, dehydrated in a graded series of ethanol then infiltrated with paraplast paraffin after transition through a graded xylene series (Calvert *et al.*, 1984). Each root was cut into 7 to 8 pieces of 10 to 12 mm length and oriented side by side in the embedding mould so that the older end of the root tissue was placed at the left and the segments were arranged with the oldest segment at the top and the youngest at the bottom (Calvert *et al.*, 1984). The blocks were serially sectioned to obtain 10µm thick sections. Slides were acid cleaned and coated with chrome alum gelatin (Pappas, 1971) prior to mounting the sections. The sections were mounted sequentially 5 to 6 to a slide and stained with tannic acid - ferric chloride and safranin - fast green (Jensen, 1962). The sections were examined by an Olympus light microscope at 10x and 40x magnifications for early infection events including curled root hairs, infection threads and cell divisions of the outer cortex. The scoring of the infection events was similar to that used by Calvert *et al.* (1984).

5.3 RESULTS

5.3.1 Co-culture of the non-nodulating mutants with the supernodulation mutant nts382 and the wild-type Bragg

Table 5.3 indicates the nodulation of the genotypes and plant growth of uninoculated plants grown at a density of six plants. jar^{-1}. The plant growth of the genotypes in the absence of nitrate indicates that the genotypes were able to grow in the absence of nitrogen for a 4 week period. The growth is indicative of the seed reserves and perhaps minimal scavenging of gaseous ammonia dissolved in water. The variable plant dry weights may be explained by the differences in seed reserves of the genotypes. When the seedlings were inoculated with *Bradyrhizobium japonicum* USDA110 the nodulation observed followed the expected pattern. At low inoculation levels (1×10^8 cells. jar^{-1}), nts382 produced supernodulation on the roots while the non-nodulation mutants failed to nodulate. Bragg, however, had the wild-type pattern of nodulation. At high inoculation (6×10^8 cells. jar^{-1}) and with increased plant densities, Bragg and nts382 nodulated in a similar manner as the low inoculation treatment but mutants nod49, nod772 and rj_1 (Lee) developed some occasional nodules. Plant dry weights increased marginally in all cases due to inoculation, except in nts382 where supernodulation was found to be deleterious to plant growth.

In the Leonard jar assembly, the root systems of the plants were tightly intertwined providing opportunities for close interactions and cross feeding of the root exudates. Sterile growth conditions were used to prevent the breakdown of exudate substances thereby allowing either maximum stimulation or suppression of nodulation to occur. Briefly, the results in Table 5.4 indicate that the nodulation characteristics of the wild-type Bragg (nodule number of about 10-20) remains the same regardless of the companion species thus indicating no interaction effects by the co-culture. In contrast, the co-culture of nts382 either with Bragg or the non-nodulation mutants indicate that nts382 developed fewer nodules per plant (Table 5.5) than in single culture (Table 5.3). Bragg and

Table 5.3 **Nodulation of soybean mutants either uninoculated or inoculated with *Bradyrhizobium japonicum* strain USDA110 at low (1×10^8 viable cells. jar^{-1}) and high (6×10^8 viable cells. jar^{-1}) of peat culture suspended in sterile distilled water.** Plants were cultured in Leonard jars at the rate of 6 plants. jar^{-1} and observed for nodulation and plant growth four weeks after germination. Data are means of 4 replications ± S.D.

Table 5.3

Treatment	Nodule no. plant^{-1}	Nodule dry wt. plant^{-1} (mg)	Root dry wt. plant^{-1} (mg)	Plant dry[a] wt. plant^{-1} (mg)
i) Uninoculated				
Bragg	0	0	143 ± 6	429 ± 38
nod139	0	0	47 ± 10	237 ± 31
rj_1(Lee)	0	0	88 ± 7	400 ± 83
nts382	0	0	91 ± 55	420 ± 125
ii) Inoculated - low				
Bragg	27 ± 9	28 ± 8	107 ± 1	515 ± 50
nod49	0	0	99 ± 14	431 ± 10
nod772	0	0	108 ± 26	521 ± 168
nod139	0	0	92 ± 16	431 ± 77
rj_1(Lee)	0	0	121 ± 29	456 ± 89
nts382	285 ± 27	57 ± 9	37 ± 5	300 ± 13
iii) Inoculated - high				
Bragg	15 ± 2.0	14 ± 0	99 ± 20	457 ± 42
nod49	1.7 ± 1.2	3.5 ± 0.7	89 ± 4	373 ± 11
nod772	0.4 ± 0	0.3 ± 0	107 ± 9	452 ± 17
nod139	0	0	99 ± 1	350 ± 34
rj_1(Lee)	2.0 ± 1.0	2.5 ± 0.7	103 ± 9	481 ± 54
nts382	289 ± 131	55 ± 6	45 ± 4	314 ± 26

[a] plant dry weight includes nodule dry weight

Table 5.4 **Co-culture of soybean nodulation mutants with the wild-type Bragg.** Plants were cultured as in Table 5.3 except that each of the 6 plants in each Leonard jar received 1 ml of 1 x 10^8 viable cells. ml^{-1}. Data are means of 4 replications ± S.D.

Table 5.4

Treatment	Nodule no. .plant^{-1}	Nodule dry wt. plant^{-1} (mg)	Root dry wt. plant^{-1} (mg)	Plant dry[b] wt. plant^{-1} (mg)
Bragg[a] plus nts382				
Bragg	14 ± 3	27 ± 7	65 ± 26	437 ± 22
nts382	127 ± 28	46 ± 13	55 ± 6	275 ± 38
Bragg plus *rj*$_1$(Lee)				
Bragg	23 ± 9	16 ± 8	71 ± 10	397 ± 62
rj$_1$(Lee)	0	0	66 ± 11	374 ± 60
Bragg plus nod49				
Bragg	8.00 ± 1.0	10.0 ± 5	66 ± 21	362 ± 62
nod49	0.08 ± 0.0	0.4 ± 0.0	68 ± 12	290 ± 30
Bragg plus nod772				
Bragg	13 ± 4	16 ± 10	65 ± 5	414 ± 26
nod772	0	0	75 ± 7	391 ± 26
Bragg plus nod139				
Bragg	10 ± 2	16 ± 4	76 ± 15	416 ± 64
nod139	0	0	65 ± 8	310 ± 13

[a] control Bragg plants in single culture grown at the rate of 6 plants. jar^{-1} and inoculated with 6 x 10^8 viable cells. jar^{-1} produced an average of 15 ± 2 nodules per plant (see Table 5.3)

[b] plant dry weight includes nodule dry weight

Table 5.5 **Co-culture of the non-nodulation mutants of the soybean cv. Bragg with the supernodulation mutant nts382.**
Plants were cultured as in Table 5.3. Data are means of 4 replications ± S.D.

Table 5.5

Treatment	Nodule no. .plant^{-1}	Nodule dry wt. plant^{-1} (mg)	Nodule no. .root dry^{-1} (mg)	Root dry wt. plant^{-1} (mg)	Plant dry[b] wt.plant^{-1} (mg)
nts382[a] plus *rj*$_1$(Lee)					
nts382	94 ± 1.2	59 ± 27	1.9 ± 0.0	51 ± 14	463 ± 39
rj$_1$(Lee)	0	0	-	85 ± 19	404 ± 44
nts382 plus nod49					
nts382	100 ± 24	42 ± 8	3.9 ± 0.6	25 ± 2	296 ± 62
nod49	0	0	-	64 ± 21	342 ± 4
nts382 plus nod772					
nts382	153 ± 34	67 ± 9	3.2 ± 0.2	48 ± 8	390 ± 22
nod772	0	0	-	99 ± 31	458 ± 88
nts382 plus nod139					
nts382	107 ± 15	49 ± 10	2.9 ± 0.6	37 ± 6	302 ± 4
nod139	0	0	-	88 ± 20	416 ± 150

a nts382 controls in single culture grown at the rate of 6 plants. jar^{-1} and inoculated with 6 x 10^8 viable cells. jar^{-1} produced 289 ± 131 nodules. plant^{-1}

b plant dry weight includes nodule dry weight

the non-nodulation mutants lowered the nodulation of nts382 being an average of 289 nodules. plant^{-1} in single culture compared to a range of 94 to 153 nodules. plant^{-1} when grown with the non-nodulators or the wild type.

Table 5.5 shows the results from co-cultures of nts382 and the non-nodulators. When compared to the Bragg/nts382 co-culture (Table 5.4), similar nodule number per plant of nts382 (about 100-150 nodules per plant) was observed when cultured with the non-nodulators. Similarly, all the non-nodulators retained this non-nodulation phenotype despite the high inoculum dose. Tables 5.4 and 5.5 support each other, indicating that the nodulation of the non-nodulators is not affected by the presence of the wild type or nts382 in their vicinity and that the non-nodulators are not cross-fed by the wild-type or nts382 root system. The lowered ratio of nodule number/ root dry weight for nts382/rj_1(Lee) co-culture (1 . 9) compared to the nts382/nod49 co-culture (3 . 9) presumably stems from the cultivar differences because rj_1 is in Lee and nod49 is in Bragg. The results shown in Tables 5.3, 5.4 and 5.5 were obtained from the same experiment. Pot experiments which were conducted in sand-vermiculite using daily irrigation of the pots with nitrogen free nutrient solution also showed no stimulation of the nodulation of the non-nodulation mutants when grown with Bragg or nts382.

5.3.2 Effect of mutant root exudate on the efficiency of *Bradyrhizobium* in nodulating wild-type soybean

The effect of preculture of *Bradyrhizobium japonicum* strain USDA110 with the exudates of the mutants nod49, nod772, nod139, rj_1(Lee), nts382 and the wild-type Bragg was studied in pouches by inoculation of Bragg test plants. The precise positioning of the nodulation region (RT-SERH) was used to moniter the rapidity of nodule initiation. It was observed that preculture of the strain USDA110 with any of the mutant exudates neither affected nodulation efficiency nor the total number of nodules on Bragg. Few nodules were formed above the

RT mark, however, a burst occurred within the first 2-3 cm below the RT. Furthermore, the total nodule number. plant^{-1} was similar (Table 5.6). If the exudate contained inhibitory substances then the nodulation sites would be substantially delayed to below the RT mark.

5.3.3 Electrophoretic root protein analysis of the mutants

One dimensional SDS-PAGE gel electrophoresis was used to study differences in the root proteins of 5 day old inoculated and uninoculated roots. The results reported in Figures 5.1(a) and (b) indicate that no detectable protein differences are observed at this stage among the various genotypes. Separate experiments on the root exudate proteins of nts382, nod49 and Bragg using one dimensional and two dimensional SDS-PAGE gel electrophoresis confirm these results (Mathews *et al.*, in preparation).

5.3.4 Rhizosphere colonization of soybean lines by CB1809

Plants were grown in a vertisol collected near Breeza, New South Wales, containing 37 mg nitrogen. kg^{-1} dry soil and free of *B. japonicum* . Observations indicated that in terms of rhizosphere populations, the disparity between inoculum dosage rates (n, n/100, 100n) narrowed with time while the rhizosphere populations increased with time. No differences were observed between the parent Bragg and the non-nodulation mutants. The supernodulation mutant nts382 supported a substantially larger population of *B. japonicum* strain CB1809 in the rhizosphere than either the parent cv. Bragg or the non-nodulation mutants. This difference was not realized until 12 days after planting as indicated in Table 5.7. The intermediate supernodulating mutant nts1116 had a larger number of *B. japonicum* strain CB1809 colonizing its root surface than either the wild-type Bragg or the non-nodulation mutants but a smaller number than nts382, indicating that it is intermediate in colonization between Bragg and nts382. With respect to the wild type and the supernodulation mutants nts382

Table 5.6 **Effect of preincubation of USDA110 in the mutant root exudate on the subsequent nodulation of cv. Bragg used as the test plant.** Tests were carried out in plastic growth pouches. The shortest emerging root hair and the root tip marks were made on the pouch prior to inoculating the seedlings with 1 ml of *B. japonicum* strain USDA110 at a titre of 1×10^8 viable cells. ml^{-1}. Plants were harvested 28 days after inoculation. Total nodule number per plant includes nodules on the lateral roots. Data are means of 20 to 47 replicate plants ± S.E.

Table 5.6

Exudate source	Test plant	Nodule number . plant^{-1} SERH - RT	Below RT	Total nodule no. plant^{-1}
none	Bragg	1.3 ± 0.3	2.9 ± 0.6	18.9 ± 1.3
Bragg	Bragg	1.4 ± 0.3	2.1 ± 0.7	17.0 ± 1.9
nod49	Bragg	0.8 ± 0.1	4.7 ± 1.3	14.8 ± 1.5
nod772	Bragg	1.7 ± 0.2	3.8 ± 0.6	19.0 ± 1.2
rj$_1$(Lee)	Bragg	1.0 ± 0.3	1.0 ± 0.5	16.3 ± 1.0
nod139	Bragg	1.0 ± 0.2	3.9 ± 0.4	18.0 ± 0.9

SERH - shortest emerging root hair
RT - root tip mark

Figure 5.1 **1-dimensional gel of the root proteins of Bragg, nod49, nod772, nod139, rj_1(Lee) and nts382.** Seeds were either (a) inoculated with *B. japonicum* strain USDA110 at planting or (b) uninoculated. Root proteins were examined 5 days after inoculation.

1. Bragg
2. nod139
3. nod49
4. nod772
5. nts382
6. rj_1(Lee)

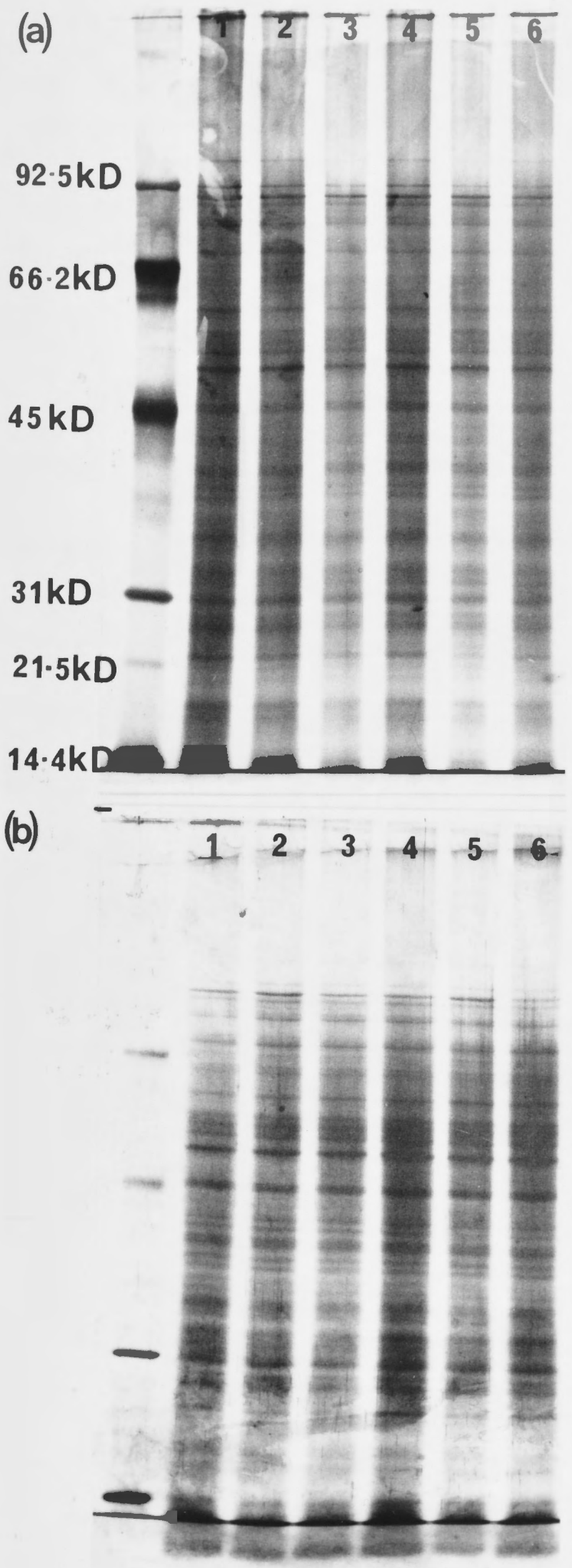

(a)
1
2
3
4
5
6
92·5kD
66·2kD
45kD
31kD
21·5kD
14·4kD
(b)
1
2
3
4
5
6

Table 5.7 **Colonization of the rhizospheres of Bragg and its induced mutants by *Bradyrhizobium japonicum* strain CB1809.** Three inoculation doses (n, n/100 and 100n) were used in the experiment. Plants were sampled 7, 12 and 17 days after emergence and the populations of rhizobia in the rhizospheres were enumerated using a tenfold serial-dilution/plant infection test with *Gycine soja* as the test plant (Brockwell *et al.*, 1975).

Table 5.7

Inoculation dose[a]	Plant genotypes					
	Bragg	nts382	nts1116	nod49	nod772	nod139
***Bradyrhizobium japonicum* strain CB1809 cell no. plant^{-1}**						
7 Days after inoculation						
n/100	1	5	1	1	1	1
n	60	60	20	85	40	30
100n	3850	215	970	5200	460	1540
12 Days after inoculation						
n/100	130	4	7	4	5	3
n	220	2630	560	2210	740	330
100n	6600	4300	31100	9420	4300	12200
17 Days after inoculation						
n/100	85	110	390	40	40	40
n	2860	42000	9400	1130	860	400
100n	10200	224000	81400	13300	8600	8600

[a] n/100 - 2.87×10^3 viable cells. ml^{-1}
n - 2.87×10^5 viable cells. ml^{-1} (normal inoculant number)
100n - 2.87×10^7 viable cells. ml^{-1}

and nts1116 there was a positive correlation between colonization and the nodulation phenotype.

5.3.5 *Bradyrhizobium japonicum* strain USDA1-110ARS attachment to soybean roots

When the mutants were inoculated with USDA1-110ARS preincubated in Bragg root exudate (2.2×10^9 viable cells. ml^{-1}), the attachment of the rhizobia was similar in all the mutants except in nod139 which had a significantly larger number of bacteria attaching to its root surface (Table 5.8). Clearly, the non-nodulation mutants are not defective in attachment. USDA1-110ARS preincubated for 18 h in half strength plant nutrient solution (2.01×10^4 cells. ml^{-1}) resulted in slightly increased number of bacteria attaching to the roots. Preincubation in Jensen's solution increased the binding of the genotypes as shown in Table 5.8. Similar results were obtained when the mutant exudate was tested for affecting attachment of the inoculant on Bragg root segments (Table 5.9).

5.3.6 Preinfection and infection of soybean mutants

Soybean genotypes inoculated with high (1×10^9 cells. ml^{-1}) and low (1×10^5 cells. ml^{-1}) cell numbers of *Bradyrhizobium japonicum* strains USDA110 and CB1795 were cultured for 5 days after inoculation. The roots cut 30 mm above the SERH and 30-40 mm below the RT mark were prepared for longitudional serial sectioning and observed by light microscopy. Nodules were visible to the naked eye at the time of fixing the roots (Table 5.10). Mutant nts382 had a higher total number of centres of cell division in the outer cortex compared to the wild-type Bragg when inoculated with *B. japonicum* strain CB1795 at both the high and low inoculations as indicated in Table 5.11. The non-nodulation mutants nod49, nod772 and rj_1(Lee) had only a few sub-epidermal cell divisions whereas nod139 did not have any at all (Tables 5.11, 5.12, 5.13). Similar trends were seen for both the actual and

Table 5.8 **Attachment of *Bradyrhizobium japonicum* strain USDA1-110ARS to soybean root segments after preincubation of the inoculant in either Bragg root exudate or half strength Jensen's plant nutrient solution for 18 h.** The initial cell numbers of USDA1-110ARS incubated in the Bragg root exudate was 3.02 x 10^9 viable cells. ml^{-1} and 3.72 x 10^9 viable cells. ml^{-1} in Jensen's plant nutrient solution . The final cell numbers of USDA1-110ARS after 18 h incubation with Bragg root exudate and Jensen's plant nutrient solution was 2.2 x 10^9 viable cells. $ml^{-1}$and 2.01 x 10^9 viable cells. ml^{-1}, respectively. The exudate was collected for 24 h. Data are the means of 30 root segments ± S.D.

Table 5.8

Plant Genotype	No. of USDA1-110ARS attached. $cm^{-1}.root^{-1}$	
	Bragg root exudate[a]	Jensen's plant nutrient solution[b]
Bragg	1139 ± 95	1651 ± 293
nts382	1931 ± 347	2404 ± 999
nod49	1185 ± 133	1040 ± 51
nod772	1137 ± 461	1512 ± 941
nod139	3861 ± 91	4734 ± 167
rj_1(Lee)	1098 ± 220	1349 ± 646

[a] *Bradyrhizobium japonicum* USDA1-110ARS was preincubated in Bragg root exudate

[b] *Bradyrhizobium japonicum* USDA1-110ARS was preincubated in Jensen's plant nutrient solution

Table 5.9 **Attachment of *Bradyrhizobium japonicum* strain USDA1-110ARS (incubated in soybean mutant root exudate for 18 h) on Bragg roots after 15 min of incubation.** USDA1-110ARS incubated in Jensen's plant nutrient solution was used as a control. Root exudate extraction and incubation of the bacteria in the exudate were similar to Table 5.8. The final viable cell number of *Bradyrhizobium japonicum* strain USDA1-110ARS incubated in the mutant root exudate was 1.30×10^9 viable cells. ml^{-1} with nod49; 0.97×10^9 viable cells. ml^{-1} with nod772; 1.30×10^9 viable cells. ml^{-1} with nts382; 1.09×10^9 viable cells .ml^{-1} with rj_1(Lee); 1.35×10^9 viable cells. ml^{-1} with nod139; and 1.28×10^9 viable cells. ml^{-1} with Jensen's plant nutrient solution. The final cell numbers were used for the attachment assay. Data are means of 30 root segments ± S.D.

Table 5.9

Treatment (root exudate + USDA1-110ARS)	No. of USDA1-110ARS attached.cm^{-1}.root^{-1}
nod49	848 ± 205
nod772	887 ± 294
nod139	939 ± 134
rj$_1$(Lee)	649 ± 96
nts382	783 ± 69
Plant nutrient solution	1373 ± 204

Table 5.10 **Number of nodules. plant^{-1} for Bragg, its induced mutants and the naturally-occurring *rj$_1$* (Lee) mutation.** Plants were inoculated with 1 ml of 1 x 10^5 and 1 x 10^9 viable cells. plant^{-1} of *Bradyrhizobium japonicum* strains CB1795 and USDA110 and cultured in plastic growth pouches. Observations were taken 5 days after inoculation. The data are means of 6 plants ± S.D.

Table 5.10

Genotype	Nodule number. plant^{-1}	
	1 x 10^5 viable cells. ml^{-1}	1 x 10^9 viable cells. ml^{-1}
	***Bradyrhizobium japonicum* strain CB1795**	
Bragg	6 ± 2	8 ± 5
nod49	0	2 ± 1
nod772	0	2 ± 1
nod139	0	*
rj_1(Lee)	*	*
nts382	44 ± 23	55 ± 12
	***Bradyrhizobium japonicum* strain USDA110**	
Bragg	2 ± 1	3 ± 2
nod49	0	0
nod772	0	0
nod139	**	*
rj_1(Lee)	*	*
nts382	53 ± 13	68 ± 11

* a nodule on one plant

** a single plant had five nodules

Table 5.11 Total sub-epidermal cell divisions per unit length of the root of the different soybean genotypes inoculated with *B.japonicum* strain CB1795 at 1 x 10^9 viable cells. ml^{-1} and 1 x 10^5 viable cells. ml^{-1}. Two root of about 80 mm length were serially sectioned and examined by light microscopy. Observations were taken 5 days after inoculation.

Table 5.11

Genotype	Total sub-epidermal cell divisions. cm^{-1}	
	***B. japonicum* strain CB1795**	
	(1×10^9 viable cells. ml^{-1})	**(1×10^5 viable cell. ml^{-1})**
Bragg	24 ± 15	14 ± 5
nod49	5 ± 4	0
nod772	5 ± 5	6 ± 9
nod139	0	0
rj_1(Lee)	0	0
nts382	41 ± 29	23 ± 9

Table 5.12 Total sub-epidermal cell divisions, actual infections and pseudoinfections. cm^{-1} of the root of the soybean mutants. Plants were inoculated with *B. japonicum* strain CB1795 at high (1×10^9 viable cells. ml^{-1}) and low (1×10^5 viable cells. ml^{-1}) dose. Root segments 70 to 80 mm in length were examined 5 days after inoculation. Two root segments were examined for each genotype.

Table 5.12

Genotype	Total sub-epidermal cell divisions. cm^{-1}		Actual infections $.cm^{-1}$		Pseudoinfections $.cm^{-1}$	
	stages of sub-epidermal cell divisions					
	I - III	IV - VIII	I - III	IV - VIII	I - III	IV - VIII
Bradyrhizobium japonicum **strain CB1795 (1 x 10^9 viable cells. ml^{-1})**						
Bragg	21.0±11.7	3.3± 2.8	8.3±1.0	1.5±0.6	12.8±11	1.9±2.2
nod49	3.4±3.3	1.1± 0.5	0.5±0.8	0.5±0.1	2.8±2.5	0.7±0.6
nod772	4.5±4.6	0.6±0.6	0	0.1±0.1	4.5±4.6	0.4±0.6
nod139	0	0	0	0	0	0
rj_1(Lee)	6.4±6.5	0.6±0.8	0	0	6.4±6.5	0.6±0.8
nts382	20.1±9.8	21.1±19.0	7.8±0.3	13.2±9.6	12.3±9.5	7.9±9.4
Bradyrhizobium japonicum **strain CB1795 (1 x 10^5 viable cells. ml^{-1})**						
Bragg	16.8±9.1	1.2±1.1	5.2±5.1	0.6±0.6	11.6±4.0	0.5±0.7
nod49	0	0	0	0	0	0
nod772	5.5±7.8	0.6±0.8	0.9±1.3	0.2±0.3	4.6±6.5	0.4±0.5
nod139	0	0	0	0	0	0
rj_1(Lee)	0	0	0	0	0	0
nts382	13.3±6.1	9.5±5.1	1.8±0.6	4.8±3.8	11.5±5.7	4.7±1.3

Table 5.13 **Total sub-epidermal cell divisions, actual and pseudoinfections. cm^{-1} of the root of the soybean mutants.** Plants were inoculated with *B. japonicum* USDA110 at high (1×10^9 viable cells. ml^{-1}) and low (1×10^5 viable cells. ml^{-1}) dose. One root segment (about 80 mm length) was examined for each genotype five days after inoculation.

Table 5.13

Genotypes	Total sub-epidermal .cm^{-1}	Actual infections .cm^{-1}	Pseudoinfections .cm^{-1}
Bradyrhizobium japonicum **strain USDA110 (1 x 10^9 viable cells. ml^{-1})**			
Bragg	19.8	10.3	9.5
nod49	0.4	0	0.4
nod772	0	0	0
nod139	0	0	0
rj$_1$(Lee)	0.4	0	0.4
nts382	21.9	7.8	14.5
Bradyrhizobium japonicum **strain USDA110 (1 x 10^5 viable cells. ml^{-1})**			
Bragg	8.6	1.7	6.3
nod49	0	0	0
nod772	0	0	0
nod139	0	0	0
rj$_1$(Lee)	0	0	0
nts382	10.0	4.5	5.4

pseudoinfections. Mutants nod49, nod772 and rj_1 (Lee) had very few actual infections but a few more pseudoinfections (Tables 5.11, 5.12, 5.13).

Of the non-nodulating mutants, only nod772 had a few sub-epidermal cell divisions at the low inoculation titre (Table 5.12). At high inoculation only nod772 and nod49 had occasional actual and pseudoinfection (Table 5.12). The supernodulation mutant had a well spread percentage of sub-epidermal cell division (actual and pseudoinfection) stages compared to Bragg which showed a skewed pattern with a major restriction of infection after stage III both at the high level of inoculation with *B. japonicum* strain CB1795 (Figure 5.2) and the low level of inoculation (Figure 5.3). Autoregulation of nodulation is manifested at about this point in Bragg. In nts382, even pseudoinfections were observed to progress frequently to advanced stages of sub-epidermal cell division (Figures 5.2 and 5.3).

A similar trend was observed in the single Bragg and nts382 root after inoculation with *B. japonicum* strain USDA110 at both high and low cell titres (Table 5.13). The non-nodulation mutants nod49 and rj_1(Lee) had a few pseudo infections at stage I while both pseudoinfections and actual infections were absent in the remaining non-nodulation mutants inoculated with the high titre (1×10^9 viable cells. ml^{-1}). At the low (1×10^5 viable cells. ml^{-1}) inoculation titre, all the non-nodulation mutants lacked any form of sub-epidermal cell divisions (Table 5.13). Both Bragg and nts382 had many infection threads above the RT mark (Table 5.14). However, the nodule number of nts382 above the RT was substantially higher than either Bragg or nts1116 (Table 5.15). The intermediate supernodulator nts1116 had less nodules above the RT mark than nts382, but more nodules than Bragg (Table 5.15). Thus, differences in nts382 and Bragg can be seen very early in nodule ontogeny.

Multiple infection threads wherein 5 to 6 infections occur as pockets are common in Bragg and nts382 as seen in Figure 5.4 (a) and (b). These multiple infection threads were more common when these genotypes were inoculated with a high cell titre *B. japonicum* strains CB1795 and USDA110. However, a few were observed at low cell titres. Sometimes the multiple infection threads

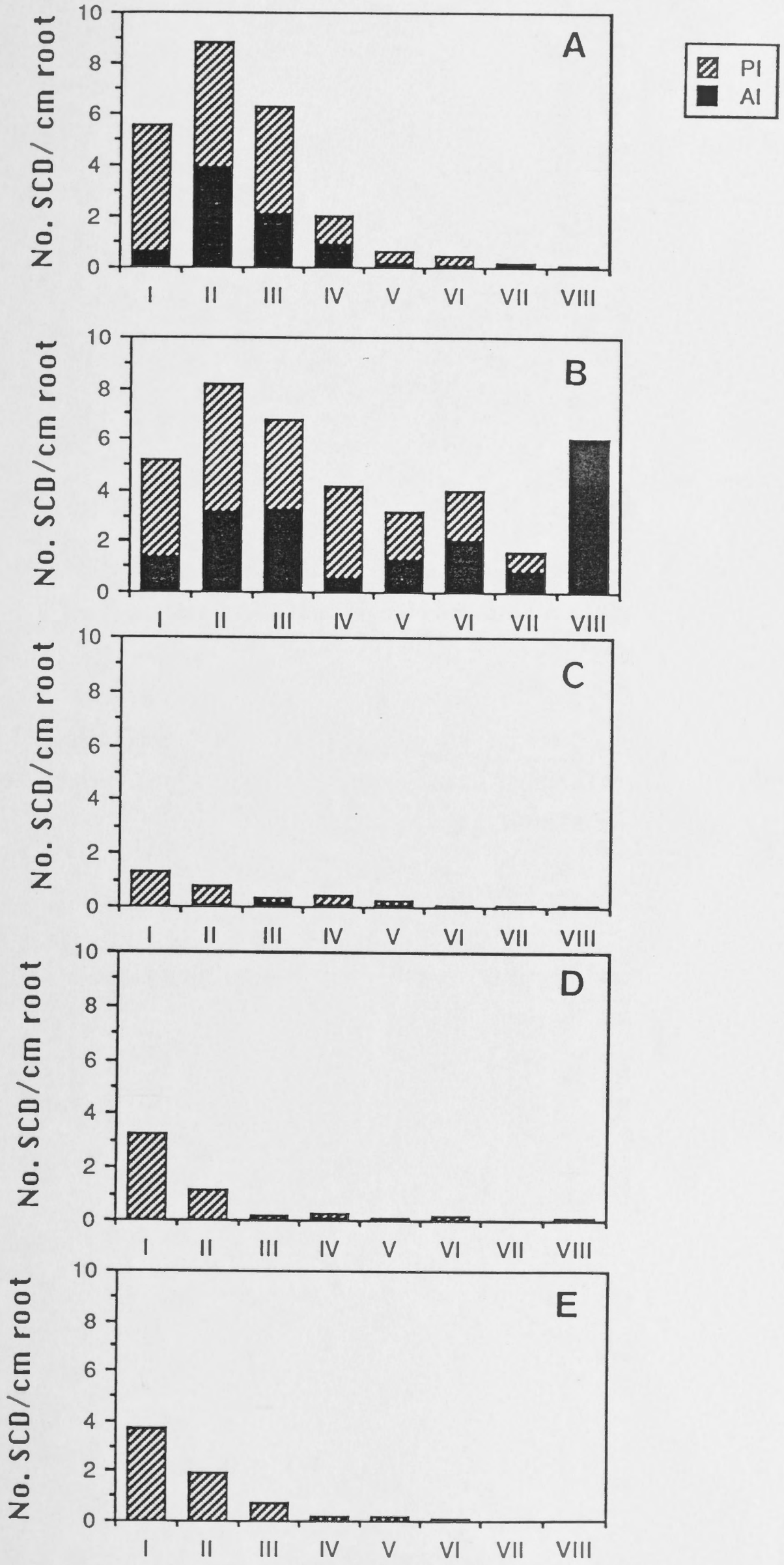
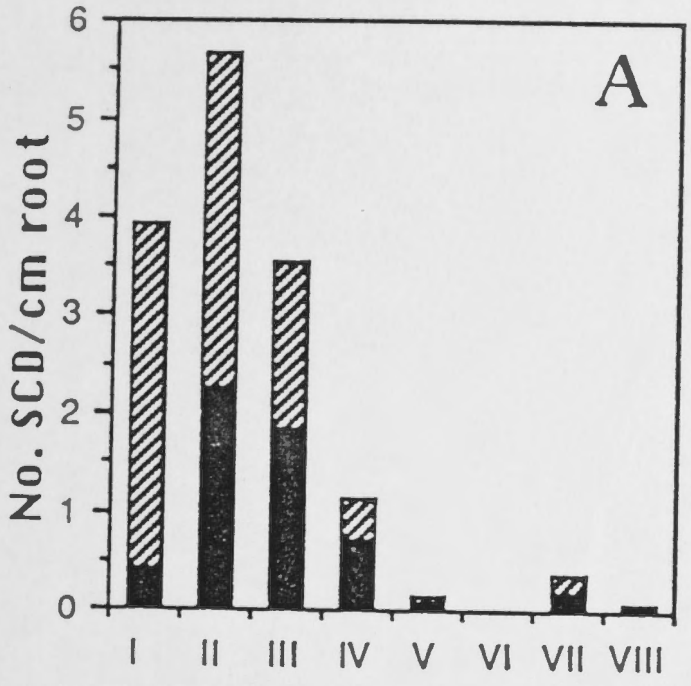
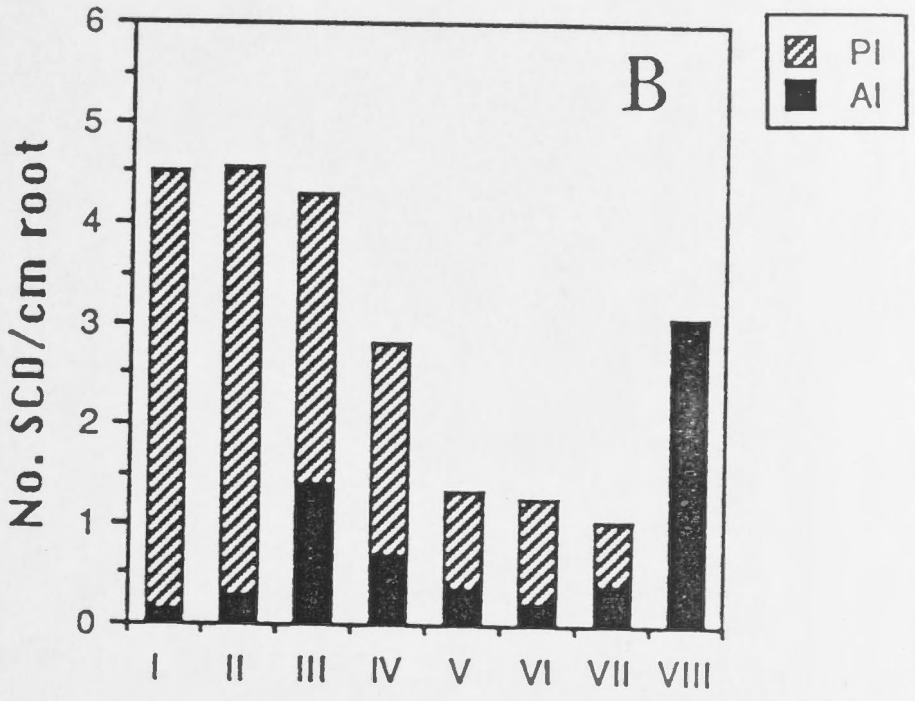
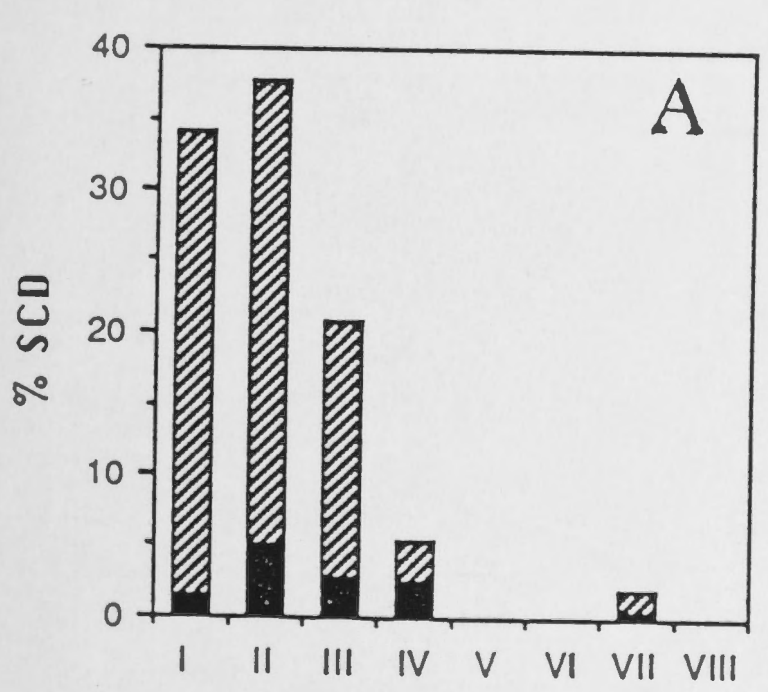

A
B
C
D
E
PI
AI
No. SCD/ cm root
No. SCD/cm root
I
II
III
IV
V
VI
VII
VIII
0
2
4
6
8
10

Figure 5.3 **The number of actual (AI) and pseudoinfections (PI) per unit root length and percentage of (sub-epidermal cell divisions) SCD of Bragg (A) and nts382(B) .** Plants were inoculated with *B. japonicum* strain CB1795 at 1 x 10^5 viable cells. ml^{-1} and cultured as in Figure 5.2. The data are means of two separate roots of about 8 cm length.


A
No. SCD/cm root
0
1
2
3
4
5
6
I
II
III
IV
V
VI
VII
VIII


B
PI
AI
No. SCD/cm root
0
1
2
3
4
5
6
I
II
III
IV
V
VI
VII
VIII


A
% SCD
0
10
20
30
40
I
II
III
IV
V
VI
VII
VIII

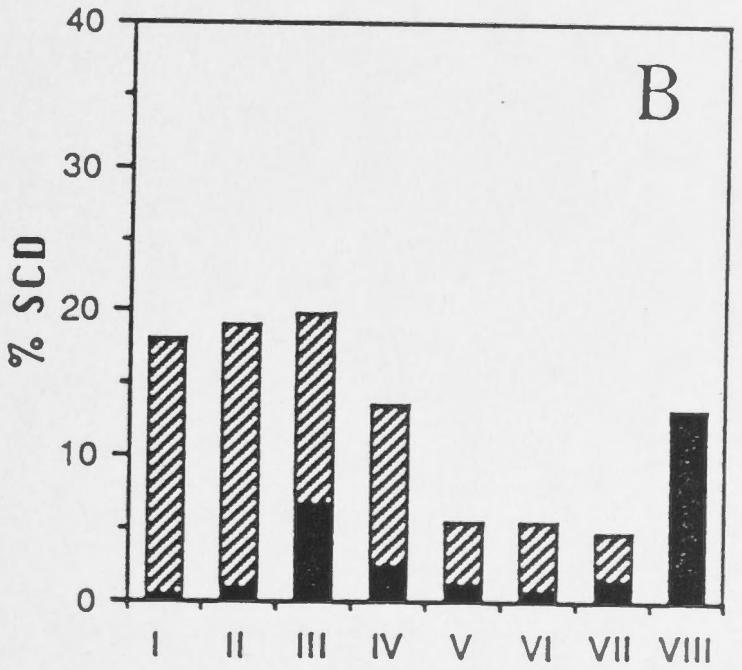

B
% SCD
0
10
20
30
40
I
II
III
IV
V
VI
VII
VIII

Table 5.14 **Actual infections per unit length of the root.** Actual infections were observed above the root tip mark made at the time of inoculating the seedlings with *B. japonicum* strain CB1795 at 1×10^9 viable cells. ml^{-1}.

Table 5.14

Genotype	Infections. cm^{-1}
Bragg	7.4 ± 4.5
nts382	15.0 ± 2.3

Table 5.15 **Nodule number. plant^{-1} on Bragg, nts1116 and nts382.** Plants were cultured in plastic growth pouches. The region of the shortest emerging root hair (SERH) and the root tip (RT) were marked prior to inoculation with 1 ml of *B. japonicum* strain USDA110 (1×10^9 viable cells. ml^{-1}). Nodulation was observed 4 weeks after inoculation. Data are means of 20 to 47 plants ± S.E.

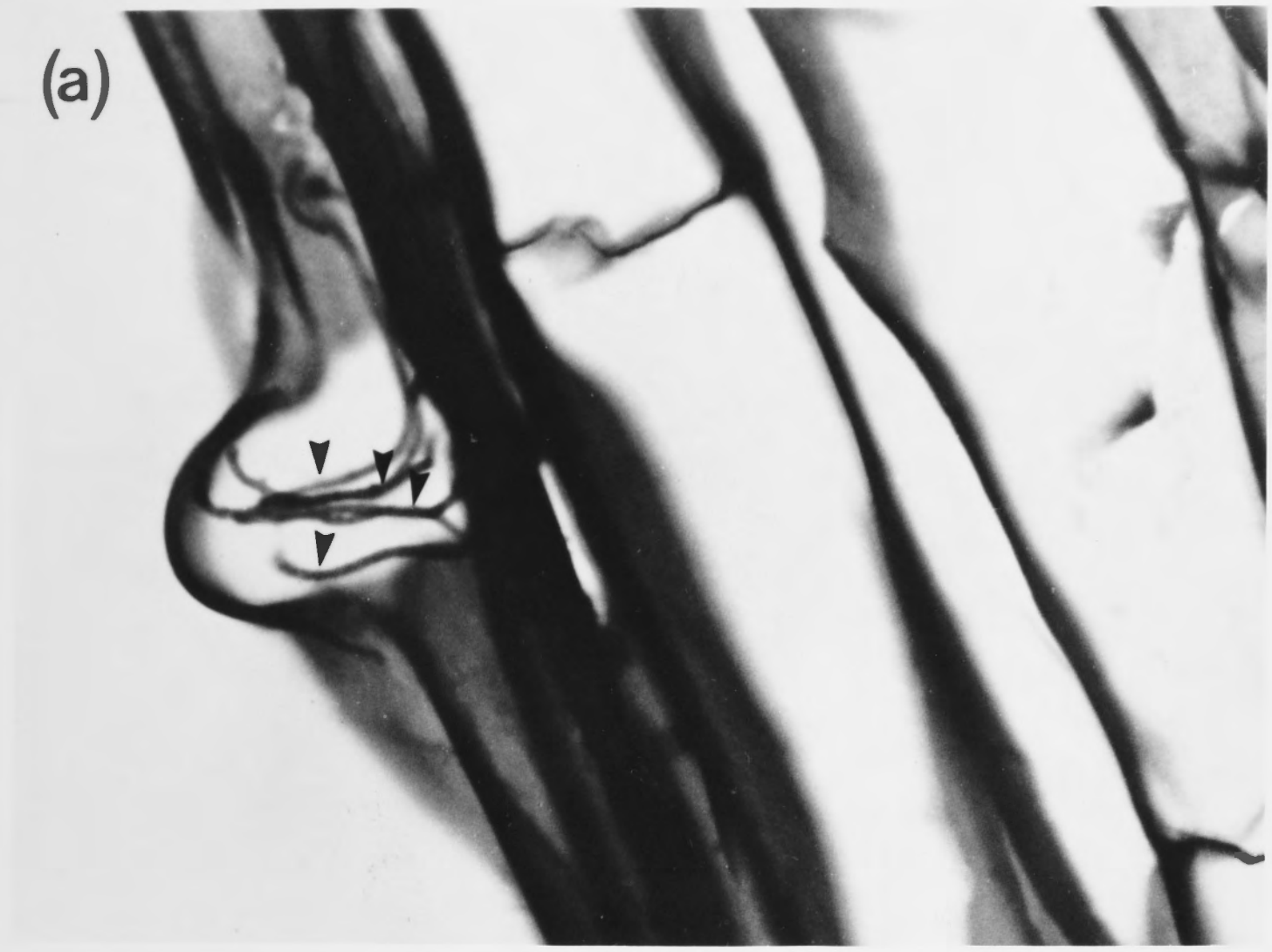
Table 5.15

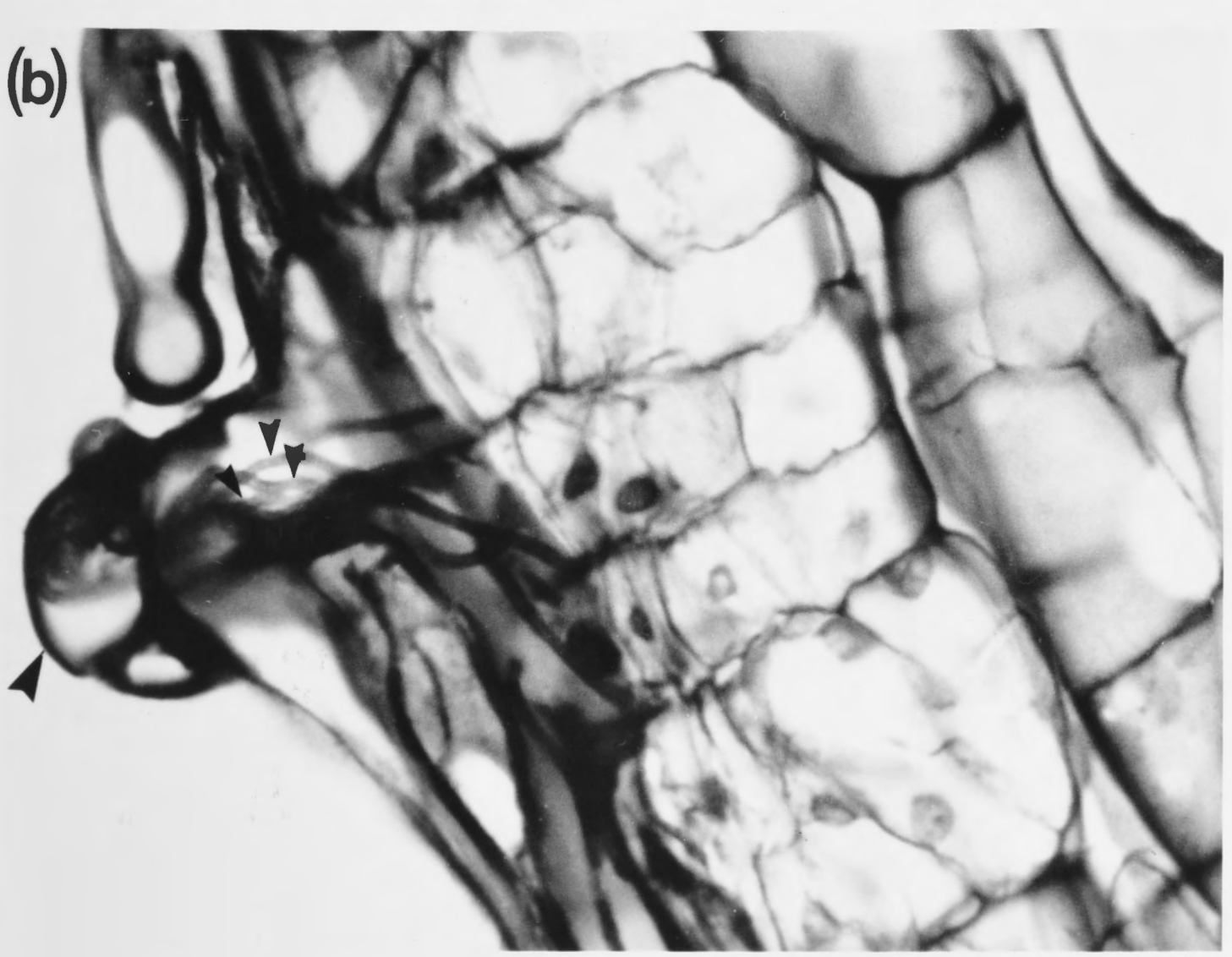
Genotype	Nodule number. plant^{-1}*	
	SERH - RT	Below RT
Bragg	1.3 ± 0.3	2.9 ± 0.6
nts1116	2.3 ± 0.4	10.9 ± 2.1
nts382	7.3 ± 1.9	17.4 ± 4.0

* only nodules on the tap root were counted

Figure 5.4 (a) **Multiple infection threads in Bragg root hair.** Note 6 infection threads in the curled root hair.The infection threads are indicated by arrows.

(b) **Multiple infection threads in nts382 root hair.** Note the penetration of the infection thread through the cortical cells indicated by the small arrows. The large arrow indicates the curled root hair.


(a)


(b)

in nts382 were associated with a long root hair curled only at the tip of the hair as shown in Figure 5.5 (a).

Sub-epidermal cell divisions were not observed in nod139 in any of the treatments. Extensive and uniform multiplication of the epidermal and cortical cells in cross section giving a corn-cob appearance of nod139 was sometimes observed in the root segments after inoculation of *B. japonicum* strains CB1795 and USDA110. This might be the cause of the abnormal root hair growth and development in nod139 resulting in the erratic distribution of root hairs on this mutant (Chapter 4).

The occasional nodule formed on the non-nodulation mutants was similar to the wild-type nodule in anatomy. It was occasionally associated with a lateral root as indicated in Figure 5.5 (b). Often these nodules were associated with a curled root hair and infection threads. At other times, the origin of infection threads in the nodule was difficult to observe. In all cases, the intercellular infection thread was always observed in the bacteroid tissue. Thus, when an infection does occur in the non-nodulating mutants, it most likely occurs in the same way as the wild type.

Figure 5.5 **(a) Multiple infection threads associated with a long root hair in nts382.** Plants were inoculated with *B. japonicum* strain CB1795. Note that the root hair is curled at the tip. The arrow indicates the infection threads.

(b) Occasional nodule initiation in mutant nod139. Plants were inoculated with *B. japonicum* strain CB1795. Note that the nodule is at the junction of a lateral root emergence point. The large arrow indicates the lateral root and the double arrow indicates the nodule.

(a)

(b)

5.4 DISCUSSION

Mutants nod49, nod139, nod772 and rj_1 (Lee) have similar root exudates to the parent Bragg on the biological level as indicated by co-culture and nodulation efficiency studies. The naturally-occurring non-nodulation variant rj_1 when previously tested for its ability to affect nodulation in co-culture with wild-type soybean was thought to excrete an inhibitory compound (Elkan, 1961). However, this result was not repeatable by Eskew and Schrader (1977). The results reported in this chapter corroborate the latter results and have also been shown to be true for the new induced non-nodulation mutant rj_6. The results also indicate an absence of cross feeding of perhaps missing substances from either the wild type or the supernodulation mutant.

Plants were grown aseptically in the Leonard jar assembly to ensure that the breakdown of either stimulatory or inhibitory substances were kept at a minimum. This system of growing plants also provides a liquid reservoir and jars do not have to be watered, thereby preventing the possibility of continued washing out of the rhizosphere compounds. The pot culture (wherein plants were watered daily) and the Leonard jar experiments gave identical results indicating that the exudates were similar. These results were further confirmed by the inoculation of precultured *Bradyrhizobium japonicum* strain USDA110 in the mutant exudate on Bragg roots (Table 5.6). There was no increase or decrease in nodulation of Bragg roots by inoculant that had been preincubated with the non-nodulation mutant exudates. Halverson and Stacey (1984a) whose method was used in the exudate preparation reported that the delay in the initiation of nodulation of mutant HS111 (i.e. nodulates below the RT mark) was overcome by preincubation of this mutant in soybean root exudate prior to inoculation. Further analysis showed that soybean seed lectin which is antigenically similar to soybean root lectin (C. Sengupta-Gopalan, *pers. comm.*) was able to duplicate the exudate effect involved in the correction of nodulation efficiency (Halverson and Stacey, 1985). However, the analysis of the root protein in the exudates of Bragg, nts382 and nod49 showed a similar pattern

both on one dimensional and two dimensional gels (Mathews *et al.*, in preparation). When radioactively (^{35}S) labelled root exudates were subjected to immunoprecipitation with the antibody to soybean lectin (SBL), similar amounts were found in nod49, nts382 and Bragg (Mathews *et al.*, in preparation). Methanol/water extracts from uninoculated seedling roots when tested for their ability to induce *nod* C-*lac* Z fusions of strain USDA123 (pEA23-2) gave similar ß-galactosidase activities in all cases, supporting the claim that nod49 and nts382 possess a similar distribution of stimulating and inhibitory substances affecting *nod* gene expression in their respective root exudates (Mathews *et al*, in preparation). However, nod49 does not respond to the ontogenetic consequence of *nod* gene expression which results in the production of sub-epidermal cell divisions. Results from the co-culture of either Bragg or the non-nodulation mutants with nts382 lowered the nodulation of the supernodulation plants by approximately 50 per cent. There are two plausible explanations for this. The wild-type or the non-nodulation plants may have competed with the smaller supernodulation plants for resources but this seems unlikely since the plant dry weights for nts382 were similar regardless of its companion plants. Alternatively, Bragg and the non-nodulation mutants may exude an inhibitor which is absent in nts382. This aspect needs to be investigated further.

Colonization and proliferation of rhizobia, a facultative symbiont, in the rhizosphere (the portion of the soil under the direct influence of the plant root system) and rhizoplane (the root surface and sometimes its adhering soil), is an important step in determining nodule initiation and competition in legumes. Biological (Damirgi *et al.*, 1967) and non-biological factors in the soil system affect the survival of the free-living rhizobia. The non-nodulation mutants nod49, nod139 and nod772 are similar in the rhizosphere colonization to the parent Bragg, indicating that they are able to elicit colonization and proliferation of *Bradyrhizobium japonicum* strain CB1809 and are not blocked at this stage of nodulation. The supernodulation mutants nts382 and nts1116 supported substantially larger populations of *B. japonicum* strain CB1809 in the rhizosphere compared to either Bragg or the non-nodulation mutants. In the enumeration of the rhizosphere population using the ten-fold serial dilution plant infection test (Brockwell *et al.*, 1975), the rhizosphere contents were dispersed into the seedling nutrient solution using a stomacher (Sharpe and Jackson,1972).

An inadequacy of this technique was thought to be the dispersion of rhizobia from the infection thread thereby resulting in the high rhizosphere population numbers of the nts382. This seems unlikely since the non-nodulation mutants which had either none or very few infection threads had the same rhizosphere colonization number as Bragg.

Rhizobia attach comparatively well to the surface of host and non-host roots alike (Chen and Phillips, 1976; Shimschick and Hebert, 1978; Pueppke, 1984; Badenoch-Jones *et al.*, 1985; Mills and Bauer, 1985; Anolles and Favelukes, 1986). The rate of bacterial attachment and the number of attaching rhizobia are considered to influence the root colonization ability and the competitive advantage of one strain of rhizobia over another (Vesper and Bauer, 1985). Attachment of *B. japonicum* strain USDA1-110ARS was studied in the infectible emergent root hair zone of the root since bradyrhizobia mainly infect and nodulate in this region of the root (Turgeon and Bauer, 1985). In addition, the number of bradyrhizobia attaching firmly to the excised zone of the root has been shown to be comparable to the number of bacteria firmly attaching to the same zone of intact roots (Vesper and Bauer, 1985). The attachment studies reported here were done using 15 min incubation which was considered adequate as the attachment process was reported to be quite rapid and almost complete in about the same time (Vesper and Bauer, 1985). Only firmly attached bradyrhizobia were counted in this assay. In terms of *B. japonicum* USDA1-110ARS attachment to the mutant roots, only minor differences were observed in the root attachment of the non-nodulation mutants and the supernodulation mutant. Mutants nod49, nod139 and rj_1 (Lee) were slightly better in attachment than the parent cv. Bragg, whereas nod772 and nts382 were similar to Bragg indicating no correlation between bacterial attachment and nodulation in these mutants.

The attachment of *B. japonicum* USDA1-110ARS preincubated either in the mutant exudate or the exudate of the wild type indicates that the non-nodulation and supernodulation mutant root exudate do not have any inhibitory or stimulatory substances which may alter attachment.

The non-nodulation mutants (nod49, nod772 and rj_1) had only few

sub-epidermal cell divisions when compared to Bragg. These, when present, were mainly pseudoinfections as they were not associated with infection threads. This indicates that these mutants are blocked at this early stage of nodulation. These mutants may require a higher concentration of some bacterial by-product or signal required to produce divisions of the outer cortex of the root. This assumption is supported by the facts that: (i) at higher cell numbers of *Bradyrhizobium* some sub-epidermal cell divisions are seen; (ii) occasional nodules are formed when plants are inoculated with high cell numbers of *Bradyrhizobium* (Chapter 4); and (iii) such occasional nodules are often concentrated at the cotton plug region of the Leonard jar assembly where rhizobia may have a better chance to multiply and infect the roots growing through the cotton plug .

Mutant nod139 (rj_6) is blocked a little earlier in the nodulation process as it does not produce any divisions of the sub-epidermal cells. It appears to be a tighter non-nodulation mutant, and is not allelic to the other non-nodulation mutants.

There appears to be a *Bradyrhizobium* strain difference in nodulation on the non-nodulation mutants. At high cell numbers of *B. japonicum* strain CB1795, more sub-epidermal cell divisions are observed on the non-nodulation mutants than with USDA110. Similarly, CB1795 better nodulates the non-nodulation mutants (Chapter 4). The wild-type Bragg is blocked at stage IV of sub-epidermal cell divisions and autoregulation could act at this point or, alteratively, it may slow the subsequent infection events. Mutant nts382 does not appear to be blocked in the autoregulation pathway since more infections and sub-epidermal cell divisions lead to nodules resulting in supernodulation. Most infections abort before they can lead to nodule formation (Munns 1968b; Dart, 1974; Nutman, 1962; Fahraeus, 1957). The high number of aborted infections may be a manifestation of the self regulation of nodule number and is called autoregulation. The supernodulation mutant can tolerate and grow in the presence of high concentration of nitrate (Carroll *et al.*, 1985a.b). This attribute may favour less abortion of infections commonly seen in the wild-type plants which do not nodulate well in the presence of added nitrogen. According to Munns (1968b), added nitrate is known to increase the number of aborted

infections. Similarly, nodule formation in soybean plant cv. Williams exposed to nitrate in plastic growth pouches led to a 2.5-fold decrease in the number of nodules formed above the RT mark which was made at the time of inoculation. Furthermore, the sub-epidermal cell divisions and infection thread formation in this region was inhibited. The reversible nature of the effects of nitrate on infection initiation was also recorded (Malik *et al.*, 1987) by exposure to nitrate. Since nts382 is a nitrate tolerant symbiosis mutant, the number of aborted infections could be less. Hence more infections could lead to the formation of nodules and supernodulation of the roots.

The non-nodulating mutant nod772 when inoculated with a low cell number of *B. japonicum* strain CB1795 (1×10^5 viable cells. ml^{-1}) had a few actual infections, confirming its leaky nature (Chapter 4). The occasional nodules on the non-nodulating mutants were normal in development and function and were similar in anatomy to the wild-type Bragg indicating that these mutants were blocked at the early stage of infection, namely, at the sub-epidermal cell division stage and not at the later stages of nodulation. The occasional nodules can be seen as a developmental escape. Occasional nodules on the non-nodulating mutants were often associated with infection threads and curled root hairs, indicating that the normal infection mechanism occurs.

Longitudinal sectioning of the root revealed the presence of multiple infection threads (as many as 5 to 6) originating in pockets in curled root hairs in Bragg and nts382. It was difficult to ascertain using light microscopy whether these infection threads arose from either a distinct or a common penetration point with subsequent branching of the thread (Figure 5. 5b). Part of the infection threads were visible in the curled region of the root hair leading into the base of the hair and into the epidermal cell which produced the root hair. This compared with results of Rao and Keister (1978) who observed 2 infection threads per root hair in soybean. In siratro, about 4 infection threads formed in the root hair cell. These radiated from the infection site then ramified the cell and penetrated the dividing cortical cells (Ridge and Rolfe, 1986).

The non-nodulating mutants did not have markedly curled root hairs (i.e. $>360^{o}$) . However, Bragg and nts382 had markedly curled root hairs (Chapter 4). Root hairs had been suggested to be the first host cells to undergo morphological and biochemical changes upon an infection by rhizobia

(Bauer, 1981). Growth hormones (indoleacetic acid and cytokinins) secreted by rhizobia have been implicated as factors responsible for root hair curling (see Vance, 1983).

Results obtained from this work indicate that sub-epidermal cell divisions occur either concomitant with or earlier than root hair curling as demonstrated by these non-nodulating mutants which can be used to dissect the nodule ontogeny. Cortical cell changes are known to occur in advance of the penetrating infection thread (Libbenga and Harkes, 1973; Newcomb *et al.,* 1979; Turgeon and Bauer, 1982). Likewise, sub-epidermal cell divisions in soybean roots can occur without hair curling and infections (Calvert *et al.,* 1984; Hirsch *et al.,* 1984; Finan *et al.,* 1985). Dudley *et al.* (1987) obtained similar results with alfalfa where sub-epidermal cell divisions were observed prior to infection thread formation. Studies on alfalfa -*Rhizobium meliloti* symbiosis indicate that sub-epidermal cell divisions occur without root hair curling. *Agrobacterium tumefaciens* carrying *nod* genes (Hirsch *et al.,* 1984; 1985) and mutants of *R. meliloti* (Finan *et al.,* 1985) can elicit the formation of nodule-like structures on the roots without markedly curled root hairs or infection threads. Therefore rhizobial invasion is not a prerequisite for inducing root sub-epidermal cell divisions. On the other hand, infection threads are always associated with sub-epidermal cell divisions suggesting that the induction of sub-epidermal cell divisions may be necessary in order to achieve an infection. At low levels of inoculation, the non-nodulating mutants had no sub-epidermal cell divisions, except for nod772 which had a few. However, at high inoculation levels, nod49, rj_1(Lee) and nod772 had sub-epidermal cell divisions but these were often at either stage I or stage II and actual infections were very rare. Thus the rate of progress of the sub-epidermal cell divisions, in addition to the availability of a developing root hair may be crucial in determining the marked root hair curling and infection thread formation.

The results obtained in this chapter also indicate that the non-nodulation mutants are altered at the early sub-epidermal cell division stages of nodulation while the supernodulation mutant nts382 is altered in the autoregulation mechanism. The non-nodulation mutants are normal in rhizosphere colonization

and rhizobial attachment to the roots when compared to the wild-type Bragg.

The non-nodulation mutants do not respond fully to the rhizobial signal and hence they do not have sub-epidermal cell divisions, root hair curling nor infection thread formation. A possible explanation for the ontogeny of sub-epidermal cell divisions, root hair curling and infection thread formation could probably be explained in terms of the following model. Compounds involved in the induction of *nod* genes in *Bradyrhizobium japonicum* have been identified as isoflavones which form a major component of soybean root extracts (Kosslak *et al.*, 1987). Induction of *nod* genes in *Bradyrhizobium* results in the synthesis of a signal which can induce sub-epidermal cell division. In response to this signal, the sub-epidermal cells which are at an advanced stage of division could synthesise a signal which induces root hair curling and infection thread formation.

CHAPTER 6

SHOOT AND ROOT FACTORS IN THE REGULATION OF NODULATION IN SOYBEAN

6.1 INTRODUCTION

Legume root nodule initiation and development are governed by signals or factors both external and internal to the two symbiotic partners. Externally, nitrate in the soil is known to restrict nodulation drastically. Internally, the mechanism of autoregulation regulates nodule formation wherein the early formed nodules systemically inhibit subsequent nodulation on the other parts of the root (Pierce and Bauer, 1983; Carroll *et al.*, 1985 a,b).

Shoot - root interactions have been implicated in the nodulation of legumes (Lawn and Brun, 1974; Lawn and Bushby, 1982; Kosslak and Bohlool, 1984). Phytohormones synthesised in the root and/or the shoot could be important in nodule initiation and development. Cytokinins and auxins are reported to be present in nodules and these compounds stimulate mitotic activity (Wipf and Cooper, 1940). Abscisic acid and gibberellins also affect nodulation when applied externally. As these are important growth regulators, they may also be involved in nodulation. Likewise, flavones or isoflavones are known to be required for the expression of *nod* genes in the microsymbiont (Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.*, 1986; Kosslak *et al.*, 1987) and their synthesis may not be restricted to the root tissue.

The wedge (or cleft) graft and the approach grafting techniques were employed to determine the effect of the scion of both the supernodulation mutant nts382 and the wild-type parent cv. Bragg on the nodulation of the four non-nodulation soybean mutants, and to determine whether the non-nodulation mutant shoots could alter the nodulation on nts382 and the wild-type roots. In other words, attempts were made to determine whether any substance(s), either inhibitory or stimulatory, formed in the scions of the plants could alter the susceptibility of the rootstocks to *Bradyrhizobium* infection. Attempts were also made to determine whether factor(s) determining supernodulation and non-nodulation are translocated through the shoot to the root or *vice versa* or restricted to specific plant tissues.

6.2 MATERIALS AND METHODS

6.2.1 Plant material and inoculant cultures

Soybean *(Glycine max* (L.) Merr.) cv. Bragg, the non-nodulation mutants nod49, nod772 and nod139, the supernodulation mutants nts382 and nts1007 (Carroll *et al.*, 1986; 1985 a,b) and rj_1 (Williams and Lynch, 1954) in the genetic background of the cv. Lee were used in these studies.

Bradyrhizobium japonicum strain USDA110 peat culture containing about 10^8 viable cells. g^{-1} was used as the inoculant strain in these experiments.

6.2.2 Plant growth conditions and the grafting technique

Plants used for grafting studies were grown in 25 cm diameter pots filled with a 2 : 1 sand vermiculite mixture at the rate of one plant per pot. Extreme care was taken to keep the pots sterile and uninoculated prior to grafting the seedlings. Glasshouse temperatures were maintained between 14^{o}C and 30^{o}C and incandescent 100W bulbs were used to extend the photoperiod to 16 h.

Ten day old seedlings were grafted using the standard reciprocal wedge grafting technique. The hypocotyl of the rootstock was severed between the cotyledons and the sand-vermiculite surface. The hypocotyl portion of the rootstock was slit from the cut end downwards for 1 cm and a wedge 1 cm long was made on the hypocotyl portion of the scion. A 2 cm polythene tubing was slipped over the rootstock and allowed to fall to the base. The wedge on the scion was inserted into the slit on the rootstock. The graft was secured tightly by moving the polythene tubing over the graft portion. The approach

grafting technique was used to determine whether the factor(s) conditioning non-nodulation were either localized in the root or systemic in the plants. Two seedlings to be grafted were grown in each pot. Ten days after germination, a 1.5 cm long slice of the stem below the cotyledon was removed from the adjoining plants. This was deep enough to expose the vascular tissue. Approximately 50 per cent of the stem was removed in the region of the excision. The stems were then firmly held together at the cut regions and secured together with teflon tape. Grafted plants were placed in a mist room under automatic intermittent misting for 7 days to prevent desiccation before the grafts had functionally joined. Plants were moved to the glasshouse and inoculated with *Bradyrhizobium japonicum* strain USDA110 at approximately 10^8 viable cells. ml^{-1} by making a slurry of the bacteria, peat and water. The pots were watered with N-free (nitrate absent) Herridge's plant nutrient solution (Herridge, 1977) twice a week, and with 5 mM KNO_3 supplemented plant nutrient solution once a week. The plants were harvested 45 days after planting and examined for nodulation and plant growth characteristics.

6.2.3 Statistical analysis

Data were analysed by the GENSTAT statistical package (Alvey *et al.*, 1977).

6.3 RESULTS

Table 6.1 indicates that non-nodulation in nod49 is controlled by the root. This is because when the shoots of either the wild-type parent cv. Bragg or nts382 were grafted onto a nod49 rootstock, the plants were non-nodulated. On the other hand, grafting nod49 onto Bragg did not alter nodulation significantly. Instead the wild-type pattern of nodulation was observed with a mean of 23 nodules. plant^{-1} compared to 26 nodules. plant^{-1} on the Bragg autograft which served as a control. Furthermore, the table shows that supernodulation in nts382 is shoot controlled. When nts382 was grafted on Bragg, the Bragg rootstock had 177 nodules. plant^{-1} compared to 26 nodules. plant^{-1} on the Bragg autograft. Irrespective of whether the scion was from a normal nodulation plant or a supernodulation plant, the nod49 rootstock always remained non-nodulated.

Similar results were obtained with nod772 which along with nod49 are allelic to *rj*$_1$ (Chapter 3) and these results are outlined in Table 6.2. Grafts using nod772 as the scion and either Bragg or nts382 as the rootstock resulted in the wild-type pattern of nodulation with 108 and121 nodules. plant $^{-1}$, respectively, and was not significantly different from the Bragg control of 116 nodules. plant^{-1}. Nodule dry weights in all cases followed a similar trend (Table 6.2). Mutant nod139 which is not allelic to *rj*$_1$ (Chapter 3) gave similar results as indicated in Table 6.3. Mutant nod139 rootstocks also remained non-nodulating with both nts382 and the Bragg shoots grafted on it. The nodule number per plant was expressed on a mg of plant dry weight basis to eliminate discrepancies associated with plants of lower vigour. No significant difference in nodule number per plant dry weight for the graft of nod139 on either Bragg or nts382 was observed compared to the Bragg controls (Table 6.3). Mutant nod139 grafted on nts1007 also had wild-type pattern of nodulation with 100 nodules. plant^{-1} compared with no nodules on the reciprocal graft. Similarly, non-nodulation in the naturally-occuring non-nodulation *rj*$_1$ (Lee) mutant is strictly determined by its root (Table 6.3).

Table 6.1 **Root control of non-nodulation in nod49 and shoot control of supernodulation in nts382.**
Ten day old seedlings were grafted using the wedge grafting technique. The grafted plants were watered with nitrate free plant nutrient solution twice a week and 5 mM KNO_3 supplemented plant nutrient solution once a week and harvested 45 days after planting. Data are means of 6 to 8 plants.

Table 6.1

Graft (Scion/Rootstock)	Nodule no. .plant^{-1}	Nodule dry wt. plant^{-1}(g)	Plant dry wt. (g)
Bragg/Bragg	26 (3.206)	0.049 (0.221)	1.622 (1.269)
nts382/nts382	284 (5.602)	0.088 (0.293)	0.532 (0.725)
Bragg/nts382	87 (4.447)	0.053 (0.278)	1.348 (1.146)
nts382/Bragg	177 (5.154)	0.127 (0.354)	1.130 (1.059)
nod49/nod49	0	0	1.177 (1.081)
nod49/nts382	69 (4.212)	0.053 (0.229)	1.432 (1.194)
nts382/nod49	0	0	0.667 (0.813)
nod49/Bragg	23 (3.100)	0.041 (0.201)	0.517 (0.718)
Bragg/nod49	0	0	0.655 (0.805)
L.S.D. (0.05)	(0.256)[a]	(0.028)[b]	(0.105)[b]

Raw data required log [a] and square root transformation [b] to satisfy assumptions for an analysis of variance. Means and L.S.D. of transformed data are shown in parantheses

Table 6.2 **Root control of non-nodulation in nod772.** Ten day old plants were grafted and inoculated with *Bradyrhizobium japonicum* strain USDA110 and harvested as in Table 6.1. Data are means of 6 to 8 plants.

Table 6.2

Graft (Scion/Rootstock)	Nodule no. .plant^{-1}	Nodule dry wt. .plant^{-1} (mg)	Plant dry wt. (mg)
Bragg/Bragg	116 (4.72)	153	3999 (62.30)
nts382/nts382	510 (6.22)	233	1047 (32.12)
nod772/nod772	0	0	1703 (48.84)
nod772/Bragg	108 (4.66)	126	3295 (57.24)
nod772/nts382	121 (4.79)	120	3295 (57.24)
nts382/nod772	0	0	1478 (38.28)
Bragg/nod772	0	0	2443 (51.47)
L.S.D. (0.05)	(0.20)[a]	(43)	(5.57)[b]

Raw data required log transformation [a] and square root transformation [b] to satisfy the assumptions for an analysis of variance for nodule number for nodulating grafts and plant dry weights of all the grafts. Means and L.S.D. of transformed data are shown in parantheses

Table 6.3 **Root control of non-nodulation in nod139 and the naturally-occurring non-nodulation mutation *rj*$_1$.** Grafts were cultured as outlined in Table 6.1. Data are means of 6 plants.

Table 6.3

Graft (Scion/Rootstock)	Nodule number . plant^{-1}	Nodule dry weight . plant^{-1} (g)	Nodule no. g pl^{-1}. dry weight (mg)	Plant dry wt.(g)
Bragg/Bragg	110 (10.42)	0.124 (0.347)	33.3 (5.74)	3.29
nts382/nts382	1541 (39.17)	0.575 (0.757)	509.9 (22.55)	3.02
nod139/Bragg	194 (13.67)	0.135 (0.363)	48.8 (6.82)	4.14
Bragg/nod139	0	0	0	3.80
nod139/nod139	0	0	0	3.02
nod139/nts382	265 (15.92)	0.128 (0.356)	57.4 (7.42)	4.58
nts382/nod139	0	0	0	3.35
rj$_1$(Lee)/Bragg	113 (10.48)	0.099 (0.308)	28.7 (5.26)	4.02
Bragg/*rj*$_1$(Lee)	0	0	0	4.04
rj$_1$(Lee)/nts382	137(11.43)	0.116 (0.337)	27.3 (5.09)	5.11
nts382/*rj*$_1$(Lee)	0	0	0	4.28
rj$_1$(Lee)/*rj*$_1$(Lee)	0	0	0	3.65
L.S.D. (0.05)	(2.8)[a]	(0.064)[a]	(1.41)[a]	0.70

Raw data required square root transformation [a] to satisfy assumptions for an analysis of variance; means and L.S.D. of transformed data are shown in parentheses

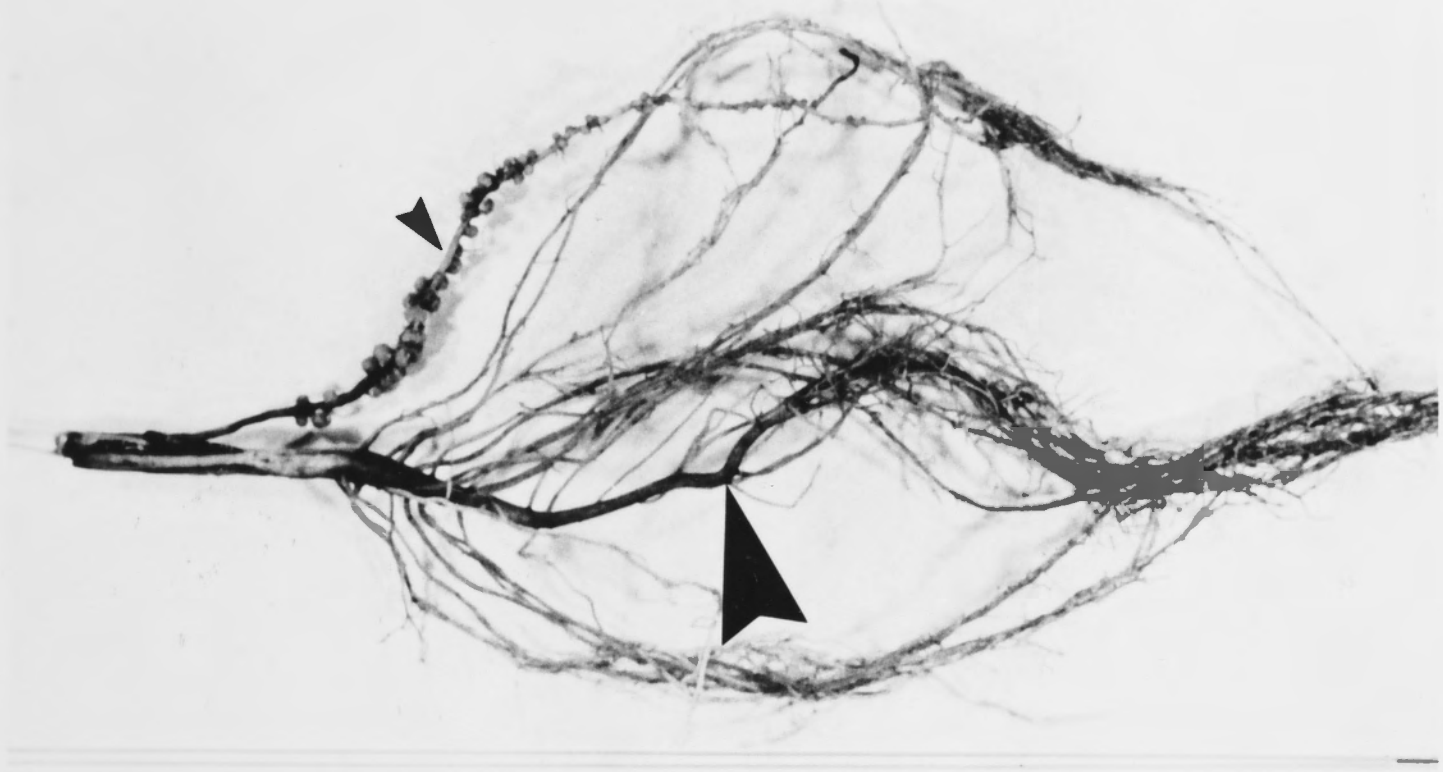
pl - plant

In another experiment several *Bradyrhizobium japonicum* strains were tested for nodulation on the grafted plants to determine whether these strains could affect their nodulation. In the case of nod49 grafted on nts382 it produced 110 nodules. plant $^{-1}$ when inoculated with USDA123; 100 nodules. plant $^{-1}$ when inoculated with USDA138-1; 221 nodules. plant^{-1} when inoculated with CB1809; and 256 nodules. plant^{-1} when inoculated with CC729. In all cases the reciprocal grafts of nts382 on nod49 produced no nodules on the nod49 rootstock. Similarly, nod49 when grafted on Bragg and inoculated with CC729 produced 42 nodules. plant^{-1} and the reciprocal grafts resulted in no nodulation on the nod49 rootstock. The grafts of the nts382 scion with the cv. Williams on the rootstock had 171 nodules. plant^{-1} compared to 50 on the graft of Williams on nts382 rootstock when inoculated with USDA123. Bragg grafted on nts382 and inoculated with CB1809 behaved in the same manner. The non-nodulation mutant nod49 grafted on Williams and inoculated with USDA110 produced 40 nodules. plant^{-1} and the reciprocal grafts had no nodules on the nod49 rootstock.

To determine whether a non-nodulation factor(s) is translocated through the graft from the root to the shoot or alternatively whether it is localized in the root, nodulation of the lateral roots arising from above the grafted portion of the plant, (i.e. from the scion) were observed. Table 6.4 indicates that the non-nodulation mutants nod49 and nod772 permitted the formation of nodules on the lateral roots arising adventitiously from the Bragg and nts382 scions. These laterals exhibited wild-type nodulation pattern with Bragg as the scion and supernodulation with nts382 as the scion as seen in Figure 6.1. In all cases, the rootstock of the non-nodulation mutants remained non-nodulated. When Bragg was grafted onto nod49 rootstock, the lateral roots arising from the Bragg scion had the wild-type pattern of nodulation with 39 nodules. lateral root^{-1} and a nodulation interval of 9.5 cm on the 23 cm total lateral root length. With nts382 as the scion, the adventitious roots arising from nts382 had a nodulation interval of 10 cm on the total 11.3 cm lateral root length. The lateral root arising from nts382 when grafted on nod772 had 284 nodules. lateral root^{-1} indicating the characteristic supernodulation character wherein nodules are seen at a higher density on the root (Table 6.4). To ascertain that the factor(s) controlling non-nodulation in nod139 is localized in the root, an experiment was conducted using the approach grafting technique. As outlined in Table 6.5, when nod139

Figure 6.1 **Nodulation of the adventitious roots arising from the nts382 and Bragg scions when nts382 and Bragg were grafted on nod49 using the wedge grafting technique.** The non-nodulating nod49 roots are indicated by the large arrow. The small arrow indicates the nodules arising from nts382 and Bragg scions. The adventitious root arising from nts382 was supernodulating and the Bragg adventitious root had the wild-type pattern of nodulation.

nts-382 Scion/Nod-49 Stock



Bragg Scion / Nod-49 stock

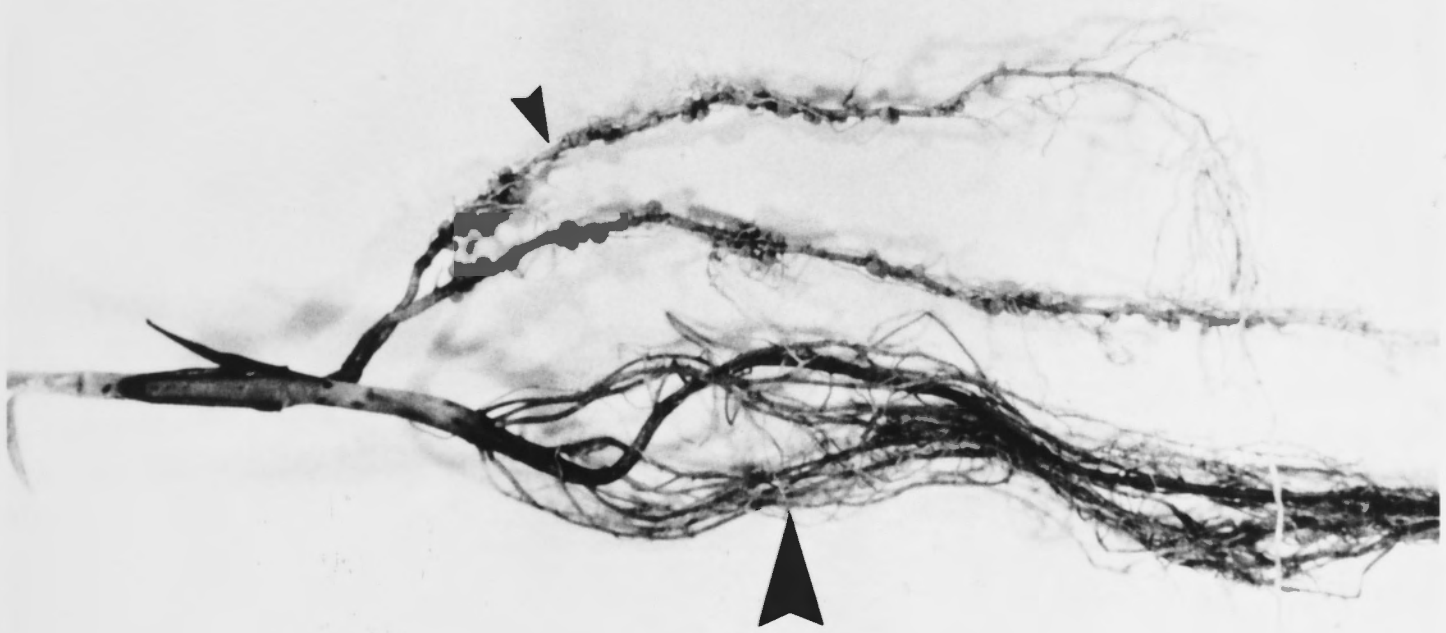


Table 6.4 **Localization of factor(s) conditioning non-nodulation in the soybean root.** Results were obtained from adventitious roots arising from the scion portion of the graft. The nodulation characteristics of the adventitious roots were observed. Each entry in the table is a mean ± S.D.

Table 6.4

Graft (Scion/Rootstock)	Total L.R. length (cm)	Nodulation interval[d] (cm)	Nodule no. .L.R^{-1}	Nodule dry wt. (mg)
Bragg/nod49[a]	28.83 ± 1.61	9.50 ± 2.65	39.33 ± 6.66	17.33 ± 2.08
nts382/nod49[b]	11.33 ± 4.45	10.00 ± 4.63	62.17 ± 13.01	11.25 ± 3.86
nts382/nod772[c]	21.50 ± 5.32	18.00 ± 2.12	283.50 ± 14.85	264.00 ± 128.69

[a] mean of 3 lateral roots
[b] mean of 6 lateral roots
[c] mean of 2 lateral roots
[d] length of root covered by nodules

L.R. - lateral root

Table 6.5 **Non-nodulation factor(s) in nod139 is not systemic.** Approach grafts were made on nod139 with either the supernodulation mutant nts382 or the parent cv. Bragg. Plants were grafted ten days after planting. The grafted plants were inoculated with *B. japonicum* strain USDA110 peat culture containing 10^8 viable cells. ml^{-1}. Data are means of 8 plants ± S.D.

Table 6.5

Graft		Nodule number. plant^{-1}	Nodule dry wt. plant^{-1}
nts382-nts382		283 ± 62	160 ± 77
Bragg-Bragg		83 ± 26	71 ± 24
Bragg-nod139	a) Bragg	86 ± 32	88 ± 31
	b) nod139	0	0
nts382-nod139	a) nts382	290 ± 99	157 ± 61
	b) nod139	0	0
nod139-nod139		0	0

was approach grafted to Bragg, the Bragg root had 86 nodules. plant^{-1} compared to 83 on the Bragg autograft. Similarly, the nts382 grafted with nod139 had 290 nodules. plant^{-1} compared to 283 on the nts382 autograft. The nodule dry weight followed a similar pattern.

6.4 DISCUSSION

The results presented in this chapter indicate that the non-nodulation phenotype in nod49, nod772, rj_1(Lee) and nod139 is strictly controlled by the root and is determined exclusively by the genotype of the rootstock. This has also been confirmed by grafting nod49 with the soybean cv. Williams. These results are in agreement with those of Tanner and Anderson (1963) for the naturally-occurring non-nodulation rj_1 mutant. The root control of non-nodulation in nod139 was confirmed by grafting to nts1007 which resulted in wild-type nodulation on the nts1007 rootstock and no nodulation on the nod139 rootstock. The results presented in Chapter 3 indicate that *nod*49 and *nod*772 are allelic to rj_1 while *nod*139 is not allelic to rj_1 and forms a new complementation group tentatively described as rj_6. The non-nodulation response in this newly described non-nodulation gene exhibits identical tissue specificity as those of the rj_1 locus (in nod49 and nod772). Likewise, non-nodulation is tissue restricted and shows no systemic effect on nodulation of either supernodulation or normal nodulation root tissue (Tables 6.4 and 6.5). In other words, the non-nodulation response cannot be translocated to either wild-type or supernodulation root tissue in a systemic fashion.

Reciprocal grafts of the non-nodulation mutants nod49 on nts382 when inoculated with different *Bradyrhizobium japonicum* strains indicated no strain dependent effect on nodulation. Lawn and Bushby (1982) also observed very little interaction effects of root, shoot and *Rhizobium* on nitrogen fixation in four Asiatic *Vigna* species.

Nutman (1949) found no mobile nodule inhibitory or stimulatory substance produced in the non-nodulation mutants of red clover. Clark (1957) and Tanner and Anderson (1963) also observed that the non-nodulating rj_1 plant did not contain a cotyledonary factor which inhibits nodulation. Degenhardt *et al.* (1976) observed in grafting experiments that neither the cotyledon nor plant top

in peas of a nodulating plant (cv. Trapper) could produce a compound which overcomes the non-nodulating character of the root of the pea cv. Afghanistan. However, Jacobsen *et al.* (1985) reported that mutant K_{24}, a non-nodulation mutant of pea, had a translocatable factor which inhibited nodulation. Hely *et al.* (1953) reported that grafting of the top of *Trifolium repens* L. (white clover) on a root of a poorly nodulating cultivar of *Trifolium ambiguum* Bieb.(Caucasian clover) resulted in plants with large healthy nodules. These observations suggest a likely role of the shoots in the regulation of nodulation.

The supernodulation of nts382 is controlled by the shoot in that nts382 shoots induce supernodulation on the wild-type rootstock. A normal autoregulatory response was, however, observed when grafted to Bragg shoots, indicating that nts382 is not altered in the root factors which affect autoregulation. Shoots from nts382 induced supernodulation on the roots of both nts1116 and the soybean cv. Williams (Delves *et al.*, 1986). On the other hand, non-nodulation is determined by the root and the factor(s) conditioning non-nodulation is localized in the root.

The shoots on the non-nodulation mutants were normal and no additional suppression of nodulation was observed with the non-nodulation shoots compared to the wild type. The roots of the non-nodulation mutants express a mutation which prevents bacterial invasion and nodulation. Although nod139 is at a different locus to rj_1, it has the same tissue specificity but a slightly different cellular response is observed as indicated in Chapter 5. The effects of the supernodulation character are realized later in the nodulation process than the blockage of nodulation in the two non-nodulating complementation groups. This may be an explanation for non-nodulation root suppression of the supernodulation shoot. In the subsequent chapter, the genetic interaction between the non-nodulation mutants and the supernodulation mutant nts382 is described in detail.

These results show that both the shoot and the root are involved in the regulation of nodulation and opens up the possibility for a detailed mechanistic analysis of the nodulation blockage in the described mutants. It will now be possible to identify the factor(s) affecting supernodulation and non-nodulation through the use of the grafting technique in conjunction with biochemical techniques.

CHAPTER 7

GENETIC INTERACTION OF THE SUPERNODULATING AND NON-NODULATING MUTANTS OF SOYBEAN

7.1 INTRODUCTION

Symbiotic nitrogen fixation in legumes is influenced by genetic (Ohlendorf, 1985) and environmental factors (Dart, 1977). Frequently, genetic factors interact to affect the phenotype. Epistasis is an example of a genetic interaction where the expression of a single trait is controlled by more than one pair of alleles (Redei, 1982). Here, a gene may mask the effect of one or more members of separate pairs of genes. The gene which masks is said to be epistatic and the masked gene is termed hypostatic. When the interaction between the products of two allele pairs results in a 9 : 3 : 4 F_2 segregation, the phenomenon is termed recessive epistasis. Thus, epistasis can be recognized by the reduction in the number of expected phenotypic classes such that two or more classes become indistinct from each other. Examples of epistatic interactions are seen in many organisms including *Drosophila*, *Triticum vulgare* (Avers, 1984) and *Trifolium repens* (Atwood and Sullivan, 1943) but these are mainly examples of physiological and biochemical epistasis. For example, in white clover *(Trifolium repens* L.*)*, there is evidence that epistasis depends on a gene-enzyme relationship in a series of successive biochemical steps in the production of hydrocyanic acid (Atwood and Sullivan, 1943) wherein the interactions are within a cell and are attributed to a biochemical pathway.

The interaction at the genetic level between the supernodulation mutant nts382 and the non-nodulation mutants nod49, nod772 *(rj_1)* and nod139 *(rj_6)* is discussed in this chapter. The non-nodulation mutants are not allelic with *nts382* as illustrated by complementation tests (Table 3.3). Backcrosses to the parent cultivar Bragg indicated a recessive nature of inheritance in the four mutant lines. Mutant nts382 is altered in the autoregulation pathway and this explains the unrestricted number of nodules on the root system. The non-nodulators, on the other hand, are unable to produce sub-epidermal cell divisions in the presence of *B. japonicum* (Chapter 5). Grafting experiments have indicated that the supernodulation character in nts382 is shoot controlled while the non-nodulation character in the non-nodulation mutants is root controlled. Furthermore, the results in Chapter 6 demonstrate the inability of the supernodulation shoot to

suppress the non-nodulation phenotype in the non-nodulation mutants. The growth studies on all of these mutants reported in Chapter 3 indicate that they are not altered in the assimilation of nitrate and that they are mutants largely specific to nodulation. The restriction in root growth of the supernodulator has been attributed to the large number of nodules on the root system since in the absence of nitrate, nts382 has similar root growth pattern to that of the wild-type Bragg plants (Day *et al.*, 1986). However, the results in Chapter 5 indicate that nts382 does have a higher number of *B. japonicum* cells in its rhizosphere and it is not known whether this aspect contributes to a decreased growth rate.

The genetic interaction between these two extreme nodulation characters was studied by analysing the progeny from crosses between nts382 and the non-nodulation mutants. The identification and confirmation of the double recessive mutants of the supernodulation and non-nodulation mutants, along with the growth characteristics of the double mutant are also described in this chapter. The results presented here are of relevance to our understanding of autoregulation, the procedure for isolating mutants defective in nodulation and the potential reasons for decreased growth associated with supernodulation.

7.2 MATERIALS AND METHODS

The F_1 seeds of the crosses between nts382 and, nod49, nod772 *(rj_1)* and nod139 *(rj_6)* (Table 3.3, Chapter 3) were grown under ambient conditions in the glasshouse to produce F_2 families. Eight to ten F_2 seeds were then planted in 25 cm diameter pots filled with a 2 : 1 mixture of sand and vermiculite. The seeds were inoculated at planting with *Bradyrhizobium japonicum* strain USDA110 (10^8 cells. $seed^{-1}$) and once again at the same rate a week after germination. The seedlings were watered with 5 mM KNO_3 supplemented Herridge's nutrient solution (Herridge, 1977) twice a week. For the first two weeks after planting, the plants received a half strength of all the nutrients, except $CaCl_2$ which was dispensed at full strength. Full strength nutrient solution was applied in the following four weeks. Plants were harvested six weeks after germination and scored for the F_2 segregation of the different nodulation phenotypes.

The selected F_2 plants were saved and selfed to obtain the F_3 seeds. Segregation ratios, nodulation and plant growth parameters of the progeny were recorded. To identify the presence of a supernodulating shoot, scions of the selected F_3 segregants were grafted onto Bragg root stocks using the wedge-grafting technique, as described in Chapter 6 . The terminal 6-8 cm of the shoots of the selected plants were grafted on 10 day old Bragg rootstocks. Bragg and nts382 shoots grafted on Bragg rootstocks served as control grafts. The pattern of nodulation on the Bragg root and plant growth parameters such as root and shoot length, and root, shoot and plant dry weight were recorded. In the wild-type pattern of nodulation, nodules were mainly seen at the crown region of the root whereas in the supernodulation pattern of growth as seen in nts382 nodules were observed on the entire root system from the top of the root to the root apex of both the primary and lateral roots.

The growth characteristics of the double mutant DM49 (containing the

nts382 and nod49 mutations in a homozygous configuration) were studied by comparing it with nts382, nod49 and Bragg lines. Plants were grown in 15 cm diameter pots with a 2 : 1 sand and vermiculite mixture and were watered either with or without nitrate and inoculant. In the nitrate treatment, plants were watered with half strength Herridge's plant nutrient solution containing 3 mM KNO_3 for the first two weeks and with full strength 6 mM KNO_3 supplemented nutrient solution for the subsequent two weeks. In the inoculation treatment, *Bradyrhizobium japonicum* strain USDA110 (10^8 cells. $seed^{-1}$) was administered at planting. Each pot received a 1000 ml of the nutrient solution daily which was adequate to wash out the accumulated residual nutrients from the previous watering. Plant growth measurements, such as root and shoot dry weight together with the plant dry weight were recorded at 7, 14, 21 and 28 days after germination.

7.3 RESULTS

7.3.1 F_2 segregation of crosses between the supernodulation mutant and non-nodulation mutants

The F_1 progeny of the 48 crosses between nts382 and nod49, nod772 *(rj_1)* and nod139 *(rj_6)* had wild-type pattern of nodulation (Table 3.3). Their root systems and the manner of nodulation on the roots were indistinguishable from the wild-type Bragg roots. Three phenotypic classes were always obtained in the F_2 families at a ratio of 9 wild type : 3 enhanced nodulation : 4 non-nodulation, instead of the classical 9 : 3 : 3 :1 ratio, thus suggesting that the nodulation phenotype of the double mutant was non-nodulation (Tables 7.1 and 7.2 and Figure 7.1). Chi-square analysis indicated a high level of significance of the observed values to the expected 9 : 3 : 4 ratio; and this conclusion was confirmed by subsequent experiments presented below.

7.3.2 Segregation of the supernodulation genotype in the F_3 and identification of double recessive mutants

Since the mutation in nts382 is recessive all the F_2 plants with enhanced nodulation were homozygous at this locus. At the other locus of interest, (i.e. the non-nodulation locus) these plants were either homozygous wild type or heterozygous for the recessive nod49 mutation. Some enhanced nodulation plants obtained in the F_2 were true breeding (Figure 7.2) while others segregated in the F_3 into three phenotypic classes in the ratio of 1 supernodulation : 2 hypernodulation : 1 non-nodulation (Figure 7.3 and Table 7.3). Although the supernodulation and hypernodulation categories were similar they could be distinguished on both the number and the pattern of nodulation on the roots. The former category which produced true breeding progeny had on average 158

Figure 7.1 **F_2 progeny segregation of a cross between nts382 and nod49.**
Three phenotypic classes were observed at the ratio of 9 wild type : 3 supernodulation : 4 non-nodulation.

9 : 3 : 4

Table 7.1 **Segregation of F_1 progeny obtained from the cross between the non-nodulation mutants nod49 and nod772 (allelic to *rj*$_1$) and the supernodulation mutant nts382 into 9 wild type : 3 supernodulation : 4 non-nodulation phenotypes.** The observed values were tested for goodness-of-fit to the expected by Chi-square analysis.

Table 7.1

Cross (♀ x ♂)	F_2 segregation Observed Wno	Sno	Nno	Expected Wno	Sno	Nno	Calculated X^2(9:3:4)
Cross No.							
			nod49 x nod382				
1.	105	40	45	106.875	35.625	47.500	0.702*
2.	66	30	35	73.688	24.563	32.750	2.160*
			nts382 x nod49				
1.	156	51	64	152.438	51.508	67.678	0.193*
2.	36	14	16	37.125	12.375	16.500	0.263*
			nod772 x nts382				
1.	83	25	38	82.125	27.375	36.600	0.277*
2.	67	20	34	68.063	22.688	30.250	0.800*
			nts382 x nod772				
1.	99	24	37	90.000	30.000	40.000	2.325*

Wno - wild type nodulation
Sno - supernodulation
Nno - non-nodulation

* calculated X^2 values were not significantly different from the expected ratio (9:3:4) at 5 per cent critical value; tabulated X^2 for 2 degrees of freedom is 5.99.

Table 7.2 **Recessive epistatic interaction between the non-nodulation gene *rj*$_6$ in nod139 and the supernodulation gene in nts382.**
The F_2 progeny segregated into 9 wild-type : 3 supernodulation : 4 non-nodulation.
Chi-square was used to test the goodness-of-fit of the observed with the expected ratios.

Table 7.2

Cross (♀ x ♂)	F_2 segregation Observed Wno	Sno	Nno	Expected Wno	Sno	Nno	Calculated X^2(9:3:4)
Cross No.							
			nod139(rj_6) x nts382				
1.	57	21	26	58.000	19.500	26.000	0.154*
2.	86	20	27	74.813	24.938	33.250	3.825*
			nts382 x nod139 (rj_6)				
1.	28	7	17	29.250	9.750	13.000	2.060*
2.	74	18	22	64.125	21.375	28.500	3.536*

Wno - wild type nodulation
Sno - supernodulation
Nno - non-nodulation

* calculated X^2 values were not significantly different from the expected ratio (9:3:4) at the 5 per cent critical value; tabulated X^2 value for 2 degrees of freedom is 5.99

Figure 7.2 **Identification of the double-recessive mutant from the progeny of a cross between the supernodulation mutant nts382 and the non-nodulation mutant nod49 .** The segregation for the F_1, F_2, F_3, F_4 and F_5 progeny are indicated. Plants were grown in 2 : 1 sand-vermiculite mix and watered with 5 mM KNO_3 supplemented plant nutrient solution twice a week.

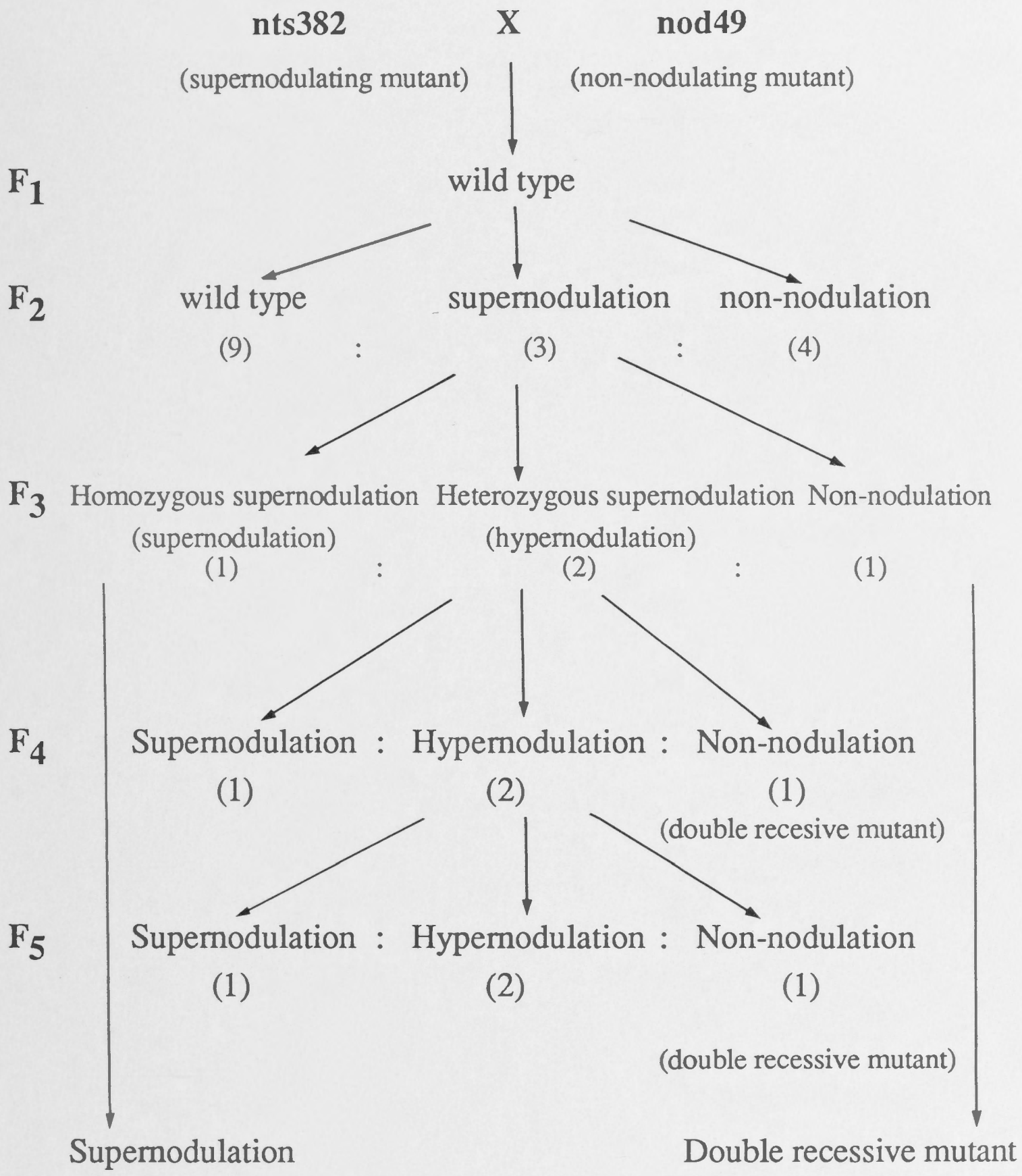

nts382
X
nod49
(supernodulating mutant)
(non-nodulating mutant)
F1
wild type
F2
wild type
supernodulation
non-nodulation
(9) : (3) : (4)
F3
Homozygous supernodulation
(supernodulation)
Heterozygous supernodulation
(hypernodulation)
Non-nodulation
(1) : (2) : (1)
F4
Supernodulation : Hypernodulation : Non-nodulation
(1) (2) (1)
(double recesive mutant)
F5
Supernodulation : Hypernodulation : Non-nodulation
(1) (2) (1)
(double recessive mutant)
Supernodulation
Double recessive mutant

Figure 7.3 **The F_3 segregation of some of the F_2 plants with enhanced nodulation into 1 supernodulation: 2 hypernodulation : 1 non-nodulation progenies.** The nodule number and pattern of nodulation of the hypernodulating progenies are different from the supernodulating progenies.


1 : 2 : 1

Table 7.3 **Segregation of the F_3 progeny derived from some of the F_2 plants with enhanced nodulation which were obtained from a cross between the supernodulation mutant nts382 and the non-nodulation mutant nod49 .** The F_3 progeny segregated at a ratio of 1 supernodulation : 2 hypernodulation : 1 non-nodulation. Chi-square was used to test the goodness-of-fit of the observed with the expected values. Other F_2 plants with enhanced nodulation bred true.

Table 7.3

Supernodulation F_3 progeny	Observed Sno	Hno	Nno	Expected Sno	Hno	Nno	Calculated X^2(1:2:1)
nts382 x nod49 cross							
1.	4	12	9	6.25	12.50	6.25	2.040*
2.	4	13	7	6.00	12.00	6.00	0.917*
3.	3	12	7	5.50	11.00	5.50	1.636*
nts382 x nod772 cross							
1.	38	68	23	32.25	64.50	32.25	3.868*
2.	26	59	26	27.25	55.50	27.25	0.441*
nts382 x nod139 cross							
1.	5	10	4	4.75	9.50	4.75	0.157*
2.	10	22	6	9.50	19.00	9.50	0.789*

Sno - supernodulation
Hno - hypernodulation
Nno - non-nodulation

*calculated X^2 values for the supernodulation F_3 phenotype were not significantly different from the expected ratio (1:2:1) at the 5 per cent critical value; tabulated X^2 for 2 degrees of freedom is 5.99

nodules. plant^{-1} distributed in a beaded manner along the entire length of the tap and lateral roots. Furthermore, all mature laterals were nodulated and were similar to the supernodulating parent nts382. These were designated as having the supernodulation phenotype. The latter had a lower mean number of nodules being 103 nodules. plant^{-1} and the nodules were distributed at a lower density on both the laterals and tap root (Figure 7.4 and Table 7.4). These were called hypernodulation progeny which on selfing gave three phenotypes at the ratio of 1 non-nodulation : 2 hypernodulation : 1 supernodulation (see Figure 7.2). From Table 7.4, it is clear that the nodule dry weight per hypernodulating plant was significantly lower than the true breeding homozygous supernodulators. The non-nodulation segregants were selected as the double recessive mutants and were designated as DM49, DM772 and DM139, for the double recessive mutants obtained from crosses between the supernodulation mutant nts382 and the non-nodulation mutants nod49, nod772 and nod139, respectively. The double recessive mutants bred true for the non-nodulation character (Figure 7.2). The root lengths of the double recessive mutants were generally larger than the supernodulation and hypernodulation segregants (Table 7.4). Similar results were obtained from the reciprocal crosses of the mutants.

7.3.3 Verification of the double recessive mutant DM49 (nts382 x nod49)

The segregants with the non-nodulation phenotype were selected in the F_3 by selfing the F_2 segregants with enhanced nodulation as described in Section 7.3.2. The shoots (scions) of these non-nodulating F_3 plants when grafted onto the wild-type Bragg (rootstock) produced supernodulation (Figure 7.5 and Table 7.5). The nodule number per plant of this graft was similar to the nts382 control grafted on Bragg (660 $\pm$ 71 vs. 478 $\pm$ 244 nodules. plant^{-1}, respectively), and the Bragg controls had 155 nodules. plant^{-1}. The root, shoot and plant dry weights of the double mutant graft were similar to the nts382 controls and much lower than the Bragg control grafts. Likewise, the root length of the Bragg rootstock with the double mutant graft (DM49) was similar to the nts382 control graft on Bragg and much lower than the Bragg control graft.

Figure 7.4 **Pattern of nodulation on the hypernodulation (a) and supernodulation (b) progeny.** Some of the enhanced nodulation plants segregated in the F_3 at a ratio of 1 supernodulation : 2 hypernodulation : 1 non-nodulation.


(a)
(b)

Table 7.4 **Nodulation and growth parameters of inoculated F_3 segregants of the F_2 plants obtained from the cross between nts382 and nod49 , nod139 and nod772.** Plants were grown in a 2 : 1 mixture of sand vermiculite and watered with 5mM KNO_3 supplemented plant nutrient solution. Plants were harvested 6 weeks after planting. Data are means of 8 plants ± S.D.

Table 7.4

Plant growth and nodulation parameters	Phenotypes: Supernodulation	Hypernodulation	Non-nodulation
	nts382 x nod49 F_3 segregants		
Root length (mm)	211.3 ± 62.0	175.0 ± 22.2	220.8 ± 25.8
Nodule no.	158.3 ± 23.1	102.6 ± 13.6	0
Nodule dry wt. (mg)	88.3 ± 10.8	39.5 ± 9.2	0
R.D.W. pl^{-1}(mg)	266.1 ± 23.5	198.5 ± 22.6	138.5 ± 64.2
S.D.W. pl^{-1}(mg)	313.0 ± 49.6	337.5 ± 46.0	353.5 ± 57.3
P.D.W. pl^{-1}(mg)	579.1 ± 68.6	536.0 ± 65.3	492.0 ± 75.4
	nts382 x nod772 F_3 segregants		
Root length (mm)	207.3 ± 53.0	196.7 ± 35.1	253.3 ± 45.0
Nodule no.	370.0 ± 162.6	216.7 ± 37.9	0
Nodule dry wt. (mg)	245.5 ± 47.4	177.7 ± 21.1	0
R.D.W. pl^{-1}(mg)	574.0 ± 9.2	440.0 ± 11.2	184.8 ± 37.6
S.D.W. pl^{-1}(mg)	641.5 ± 16.3	501.3 ± 34.2	410.7 ± 46.1
P.D.W. pl^{-1}(mg)	1215.5 ± 25.5	941.3 ± 39.0	595.5 ± 41.6
	nts382 x nod139 F_3 segregants		
Root length (mm)	235.0 ± 7.1	262.5 ± 26.5	320.0 ± 17.3
Nodule no.	420.0 ± 70.7	300.0 ± 60.6	0
Nodule dry wt. (mg)	253.3 ± 27.5	211.0 ± 53.7	0
R.D.W. pl^{-1}(mg)	347.0 ± 8.8	291.5 ± 65.8	258.8 ± 46.6
S.D.W. pl^{-1}(mg)	570.3 ± 48.1	472.0 ± 60.8	507.5 ± 122.9
P.D.W. pl^{-1}(mg)	743.8 ± 191.8	763.5 ± 126.6	766.3 ± 153.2

R.D.W. - root dry weight (includes nodule dry weight)
S.D.W. - shoot dry weight
P.D.W. - plant dry weight

Figure 7.5 **Nodulation pattern on the Bragg rootstocks grafted with either Bragg, nts382 or the double recessive mutant DM49 scions.** The double recessive mutant (obtained from the cross between nts382 x nod49) supernodulated the Bragg rootstock.


Bragg
nts 382
Double mutant
(nts382 x nod49)
Grafted on Bragg rootstock

Table 7.5 **Nodule number and plant growth characteristics of the grafts of the double mutant shoots grafted on Bragg rootstocks.** nts382 and Bragg shoots grafted on Bragg rootstocks served as control grafts. The terminal portion of the shoots were grafted on 10 day old Bragg roots. The grafts were watered with 5 mM KNO_3 supplemented plant nutrient solution twice a week. Data are mean of 8 plants ± S.D.

Table 7.5

Plant growth and nodulation parameters	Graft (Scion/Rootstock)		
	Bragg/Bragg	nts382/Bragg	DM49/Bragg
Root length (mm)	441 ± 41	263 ± 74	275 ± 57
Shoot length (mm)	280 ± 20	252 ± 5	290 ± 50
Nod. no. pl^{-1}	155 ± 33	478 ± 244	660 ± 71
Nod. dry wt. pl^{-1} (mg)	103 ± 25	140 ± 62	173 ± 37
R.D.W. pl^{-1} (mg)	539 ± 67	259 ± 123	302 ± 52
S.D.W. pl^{-1} (mg)	1670 ± 320	619 ± 193	993 ± 103
P.D.W. pl^{-1} (mg)	2210 ± 381	878 ± 316	1295 ± 141

DM49 - double recessive mutant (obtained from nts382 x nod49 cross)

Nod. - nodule
R.D.W - root dry weight (includes nodule dry weight)
S.D.W. - shoot dry weight
P.D.W. - plant dry weight
pl - plant

7.3.4 Growth characteristics of the double recessive mutant DM49 (nts382 x nod49)

Mutant DM49 the double recessive mutant obtained from crosses between nts382 and nod49 was compared with nts382, nod49 and the wild-type Bragg for its growth characteristics (see Figures 7.6, 7.7, 7.8 and 7.9). When the plants depended solely on nitrate as their nitrogen source (i.e. cultured with 6 mM KNO_3 supplemented plant nutrient solution and uninoculated), the plant weight of the double mutant was similar to nod49 while Bragg had a slightly larger total plant weight indicating that the double mutant was similar to nod49 in the assimilation of nitrate as seen in Figure 7.6(a). However, from Figure 7.6 (b), it is evident that at 28 days after planting when the genotypes were inoculated and treated with nitrate, the plant dry weight of DM49 was lower than nod49. When the genotypes were solely dependent on symbiotically fixed nitrogen, the plant dry weight of all the lines decreased substantially. Mutant nod49 and DM49 had similar plant dry weights which were slightly higher than nts382 as indicated in Figure 7.6(c). Figures 7.7(a),(b) and (c) indicate that the root length of DM49 was higher than nts382 but similar to Bragg in all the treatments, and that nod49 had a larger root length compared to Bragg. In the nitrate treatment, the root dry weight of the DM49 was higher than nts382. Mutant nod49 had similar root dry weight to Bragg as seen in Figure 7.8(a) whereas when the plants were treated with nitrate and inoculated, the root dry weight of nts382 was higher than that for DM49. Again, nod49 had a higher root dry weight than Bragg as shown in Figure 7.8(b). In the inoculation treatment also, DM49 had a higher root dry weight compared to nts382 and was similar to nod49 indicated in Figure 7.8(c). The shoot dry weights of all the genotypes followed a similar trend to the plant dry weight in all the treatments as shown in Figures 7.9(a), (b) and (c). Likewise, the shoot dry weight of the genotypes were similar to the plant dry weight obtained in the treatment where the genotypes were inoculated and treated with nitrate. That is, four weeks after planting, the shoot and plant dry weight of nod49 was similar to Bragg, and DM49 was slightly smaller than nod49 but still larger than nts382 indicating that supernodulation may not be the sole reason for decreased plant growth of nts382. In addition, at four weeks after planting nts382 would have considerably more *B. japonicum* in the rhizosphere (Chapter 5).

Figure 7.6 **Plant growth characteristics of Bragg, nod49, the double recessive mutant DM49 (obtained from a cross between nts382 and nod49) and nts382.**

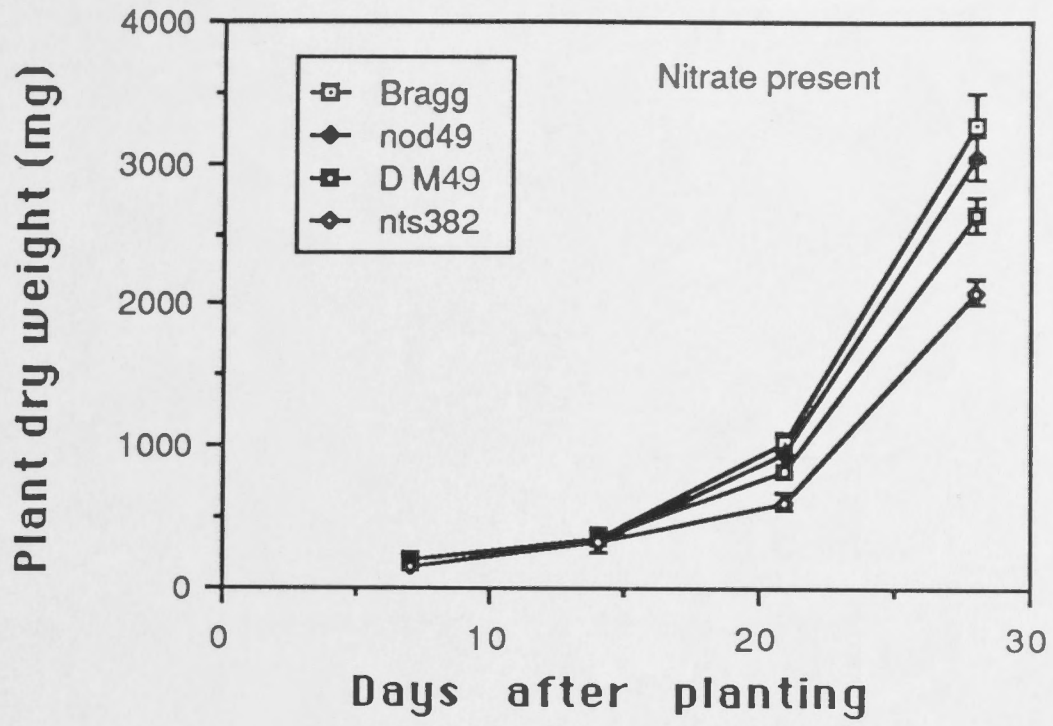
(a) plants were grown on 6 mM KNO_3 supplemented plant nutrient solution and uninoculated,

(b) plants were grown on 6 mM KNO_3 supplemented plant nurtrient solution and inoculated, and

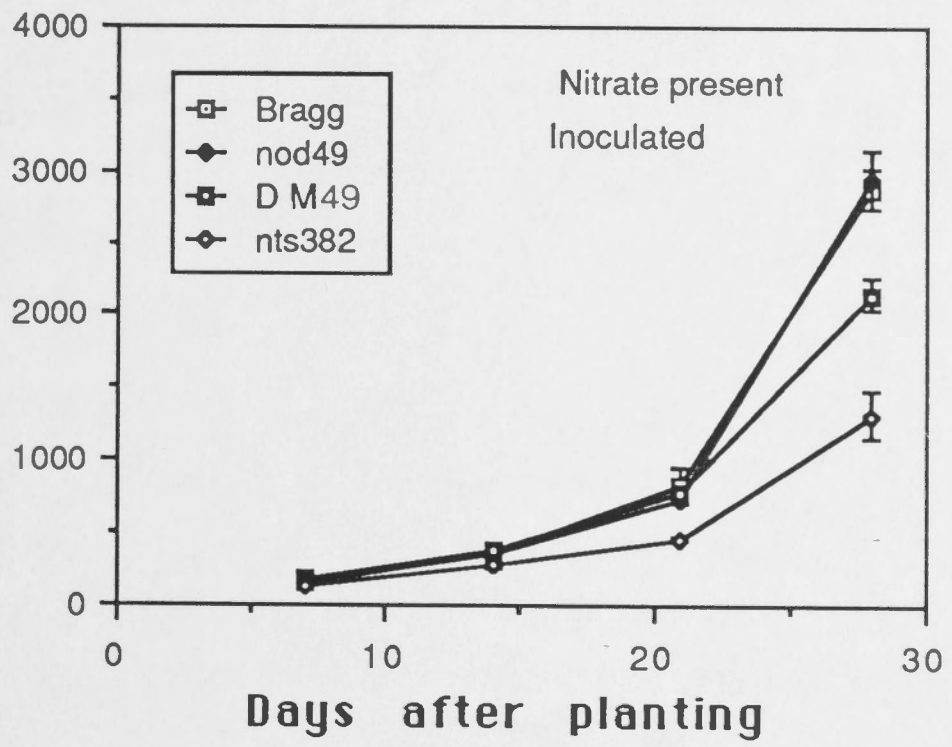
(c) plants were grown in the absence of nitrate and inoculated.

Seeds were inoculated with *B. japonicum* strain USDA110 at 10^8 viable cells. pot^{-1}. Plants were harvested 7, 14, 21 and 28 days after planting. Data are means of 8 plants ± S.D.

(a)


Plant dry weight (mg)
4000
3000
2000
1000
0
0
10
20
30
Days after planting
Bragg
nod49
D M49
nts382
Nitrate present

(b)


Plant dry weight (mg)
4000
3000
2000
1000
0
0
10
20
30
Days after planting
Bragg
nod49
D M49
nts382
Nitrate present
Inoculated

(c)

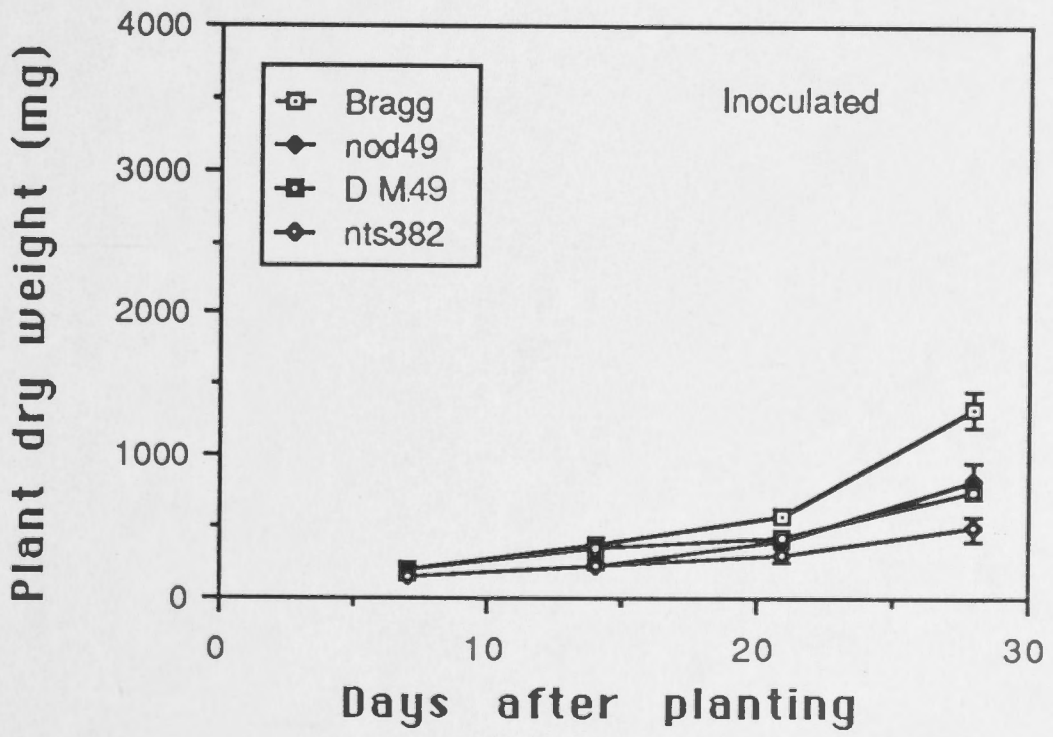
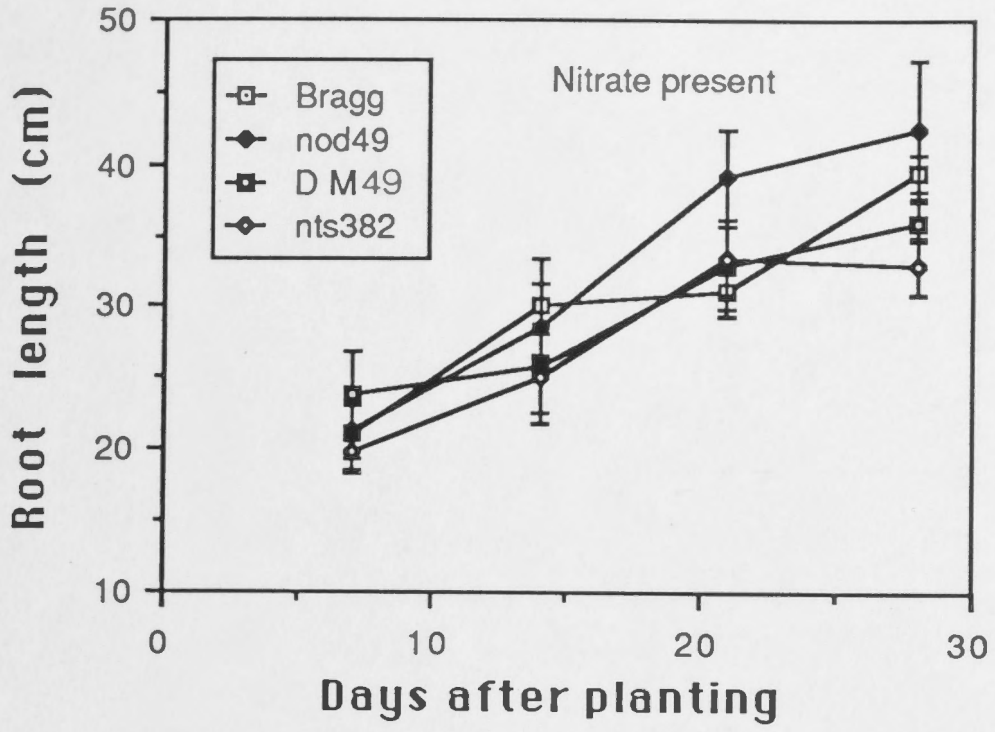
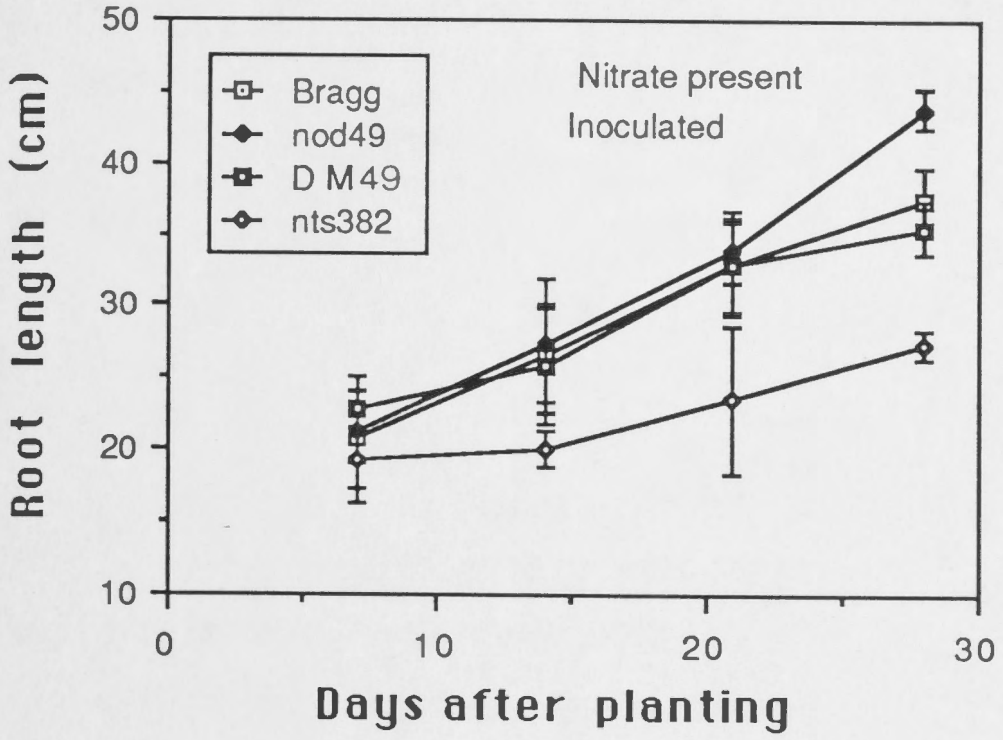

Plant dry weight (mg)
4000
3000
2000
1000
0
0
10
20
30
Days after planting
Bragg
nod49
D M49
nts382
Inoculated

Figure 7.7 **Root length of Bragg, nod49, the double recessive mutant DM 49 (obtained from a cross between nts382 and nod49) and nts382.** The treatments were as in Figure 7.6. Data are means of 8 plants ± S.D.

(a)


Root length (cm)
50
40
30
20
10
0
10
20
30
Days after planting
Bragg
nod49
D M49
nts382
Nitrate present

(b)


Root length (cm)
50
40
30
20
10
0
10
20
30
Days after planting
Bragg
nod49
D M49
nts382
Nitrate present
Inoculated

(c)

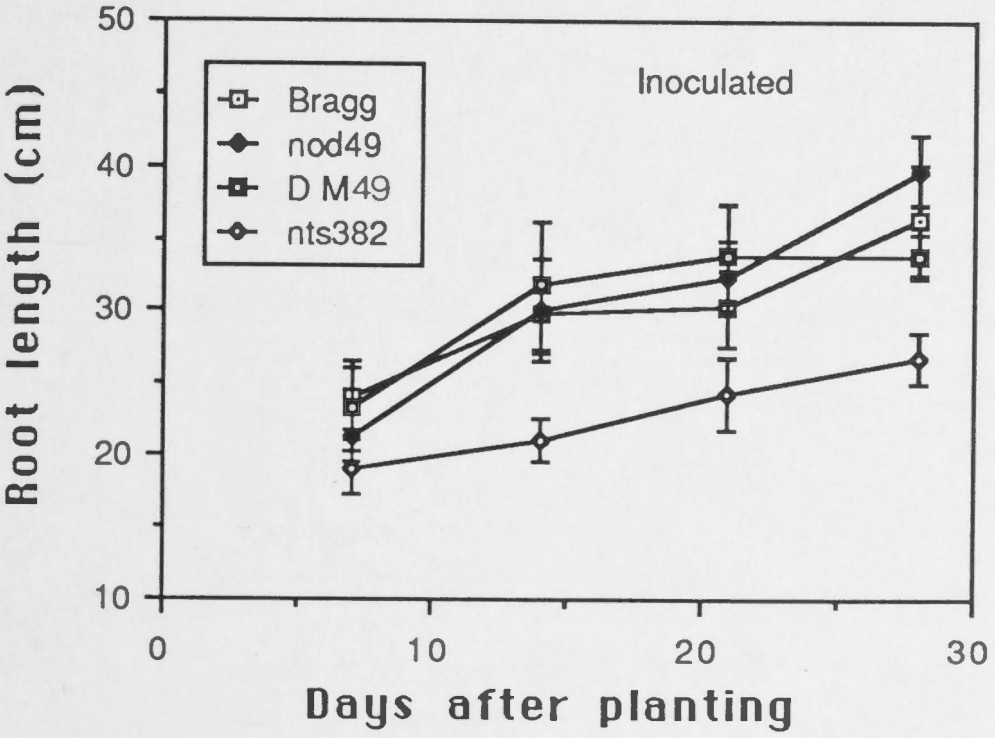
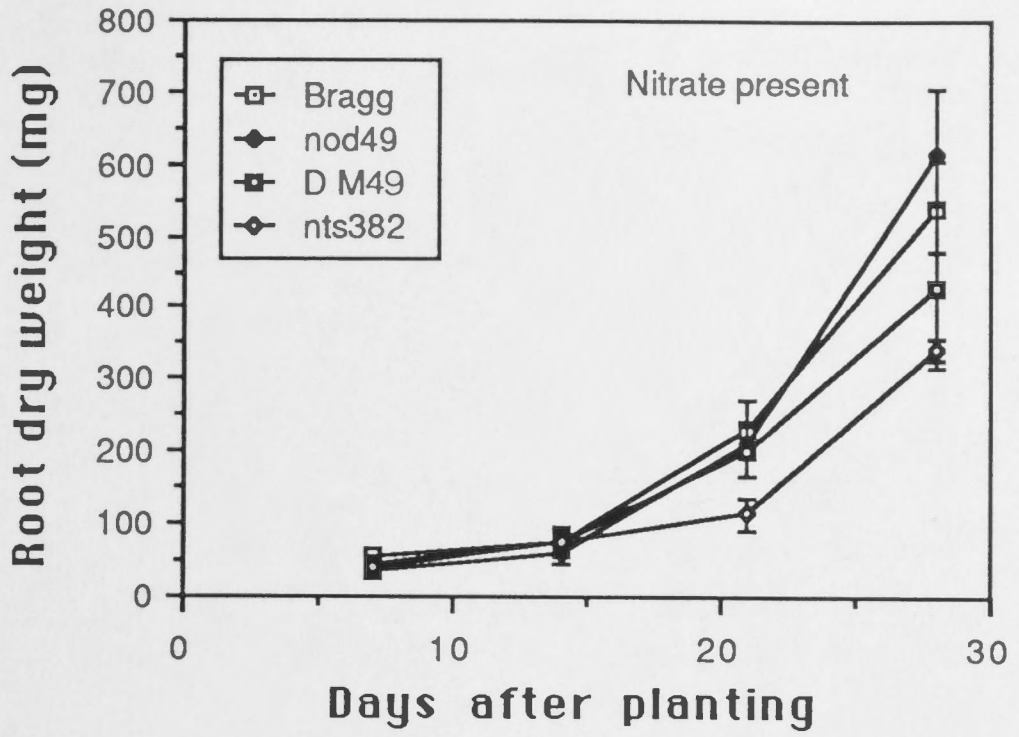
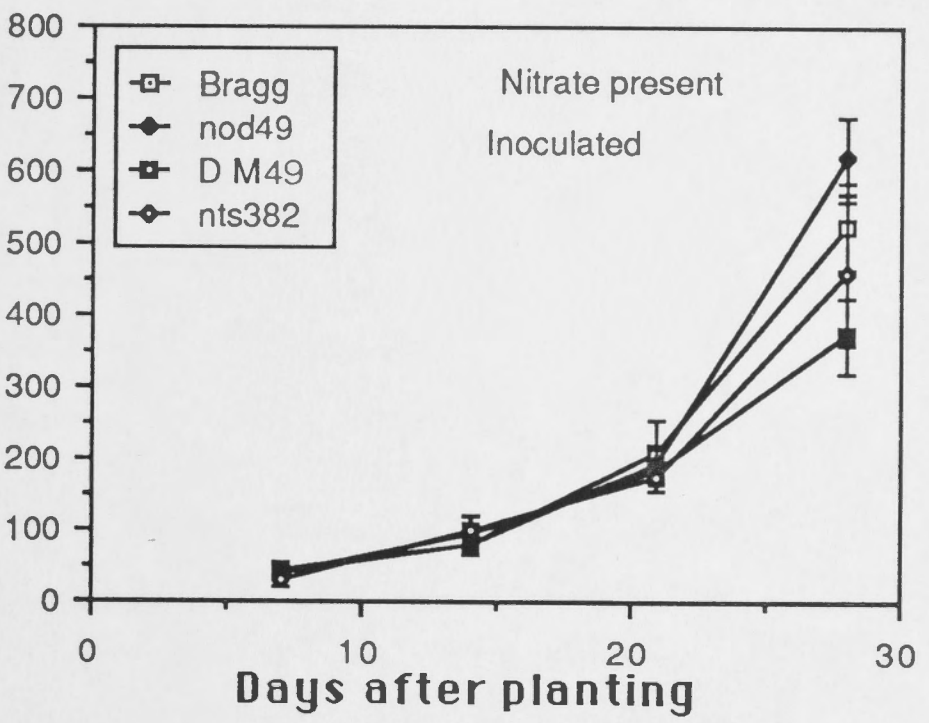

Root length (cm)
50
40
30
20
10
0
10
20
30
Days after planting
Bragg
nod49
D M49
nts382
Inoculated

Figure 7.8 **Root dry weight of Bragg, nod49, the double recessive mutant DM49 (obtained from crosses between nts382 and nod49) and nts382.** The treatments were as in Table 7.6. Plants were harvested 7, 14, 21 and 28 days after planting. Data are means of 8 plants ± S.D.

(a)


Root dry weight (mg)
800
700
600
500
400
300
200
100
0
Bragg
nod49
D M49
nts382
Nitrate present
0
10
20
30
Days after planting

(b)


Root dry weight (mg)
800
700
600
500
400
300
200
100
0
Bragg
nod49
D M49
nts382
Nitrate present
Inoculated
0
10
20
30
Days after planting

(c)

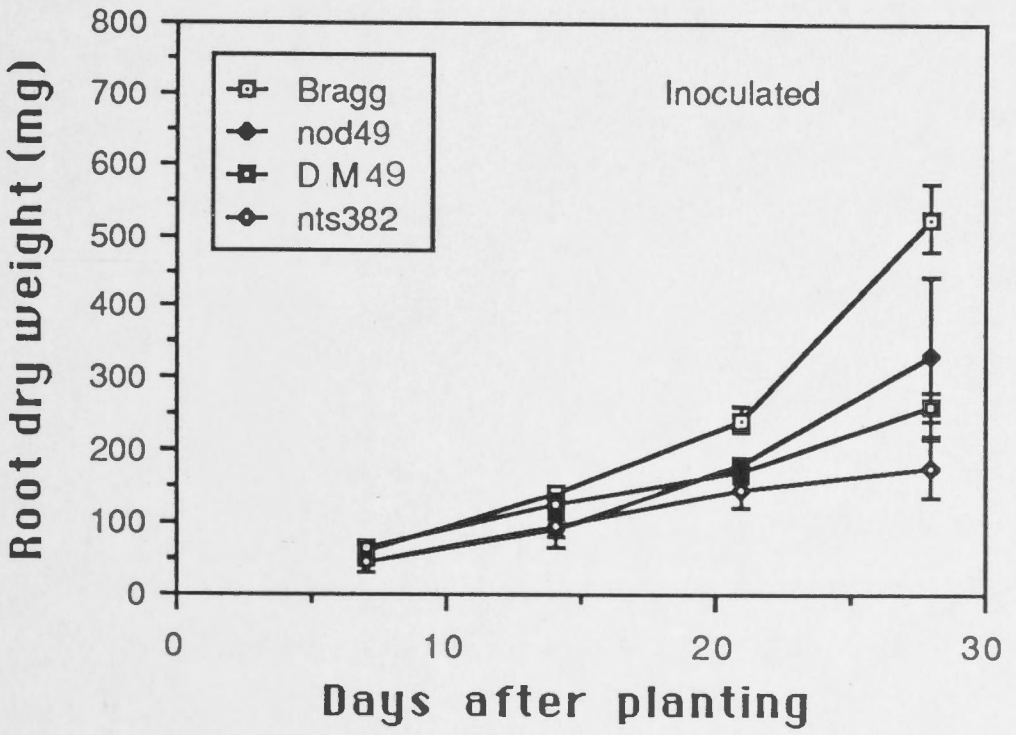
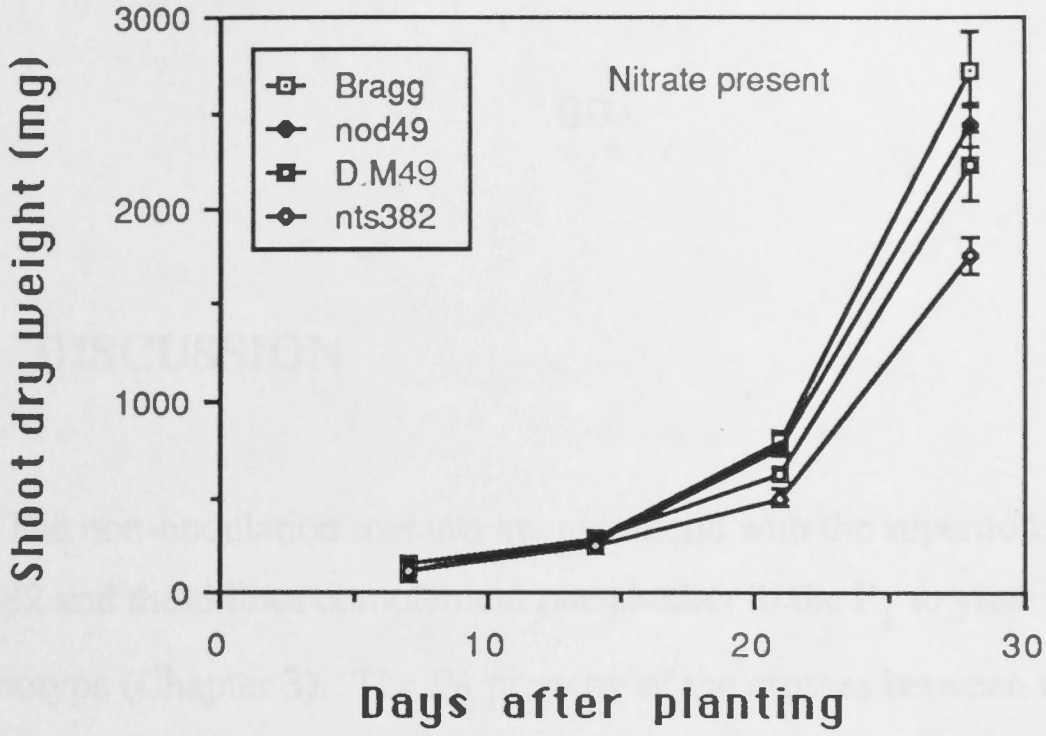
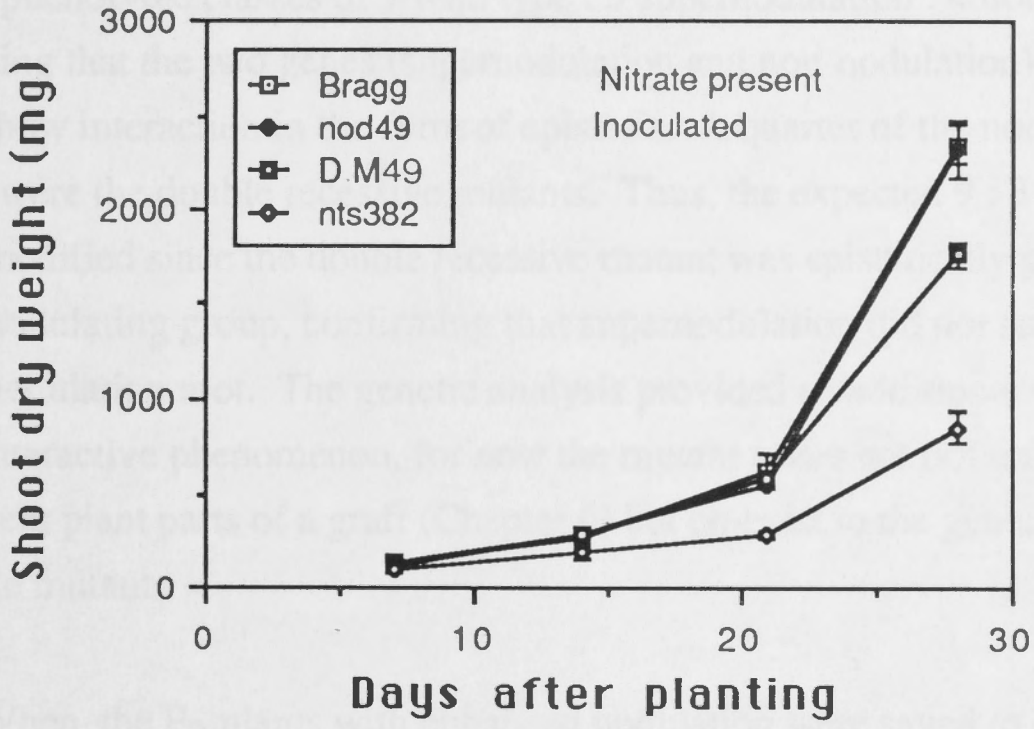

Root dry weight (mg)
800
700
600
500
400
300
200
100
0
Bragg
nod49
D M49
nts382
Inoculated
0
10
20
30
Days after planting

Figure 7.9 **Shoot dry weight of Bragg, nod49 and the double recessive mutant DM49 (obtained from crosses between nts382 and nod49) and Bragg.** Plants were cultured as in Figure 7.6 and harvested at 7, 14, 21 and 28 days after planting.
Data are means of 8 plants ± S.D.

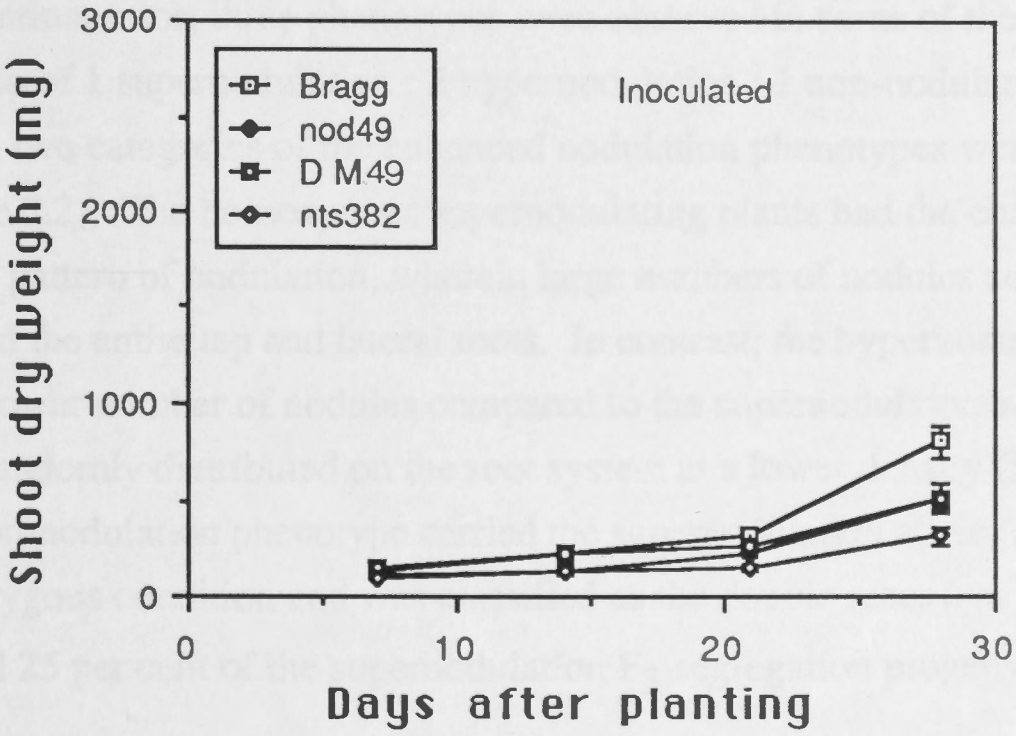
(a)


Shoot dry weight (mg)
3000
2000
1000
0
Bragg
nod49
D M49
nts382
Nitrate present
0
10
20
30
Days after planting

(b)


Shoot dry weight (mg)
3000
2000
1000
0
Bragg
nod49
D M49
nts382
Nitrate present
Inoculated
0
10
20
30
Days after planting

(c)


Shoot dry weight (mg)
3000
2000
1000
0
Bragg
nod49
D M49
nts382
Inoculated
0
10
20
30
Days after planting

7.4 DISCUSSION

The non-nodulation mutants are not allelic with the supernodulation mutant nts382 and these lines complement one another in the F_1 to yield the wild-type phenotype (Chapter 3). The F_2 progeny of the crosses between the supernodulation mutant nts382 and the non-nodulation mutants segregated into three phenotypic classes of 9 wild type : 3 supernodulation : 4 non-nodulation, denoting that the two genes (supernodulation and non-nodulation) are unlinked and show interaction in the form of epistasis. A quarter of the non-nodulation class were the double recessive mutants. Thus, the expected 9 : 3 : 3 : 1 ratio was modified since the double recessive mutant was epistatically placed with the non-nodulating group, confirming that supernodulation did not suppress the non-nodulation root. The genetic analysis provided an additional verification of this interactive phenomenon, for now the mutant genes are not only expressed in different plant parts of a graft (Chapter 6) but co-exist in the genome of the double mutant.

When the F_2 plants with enhanced nodulation were saved to obtain the F_3 segregation ratios, three phenotypes were observed in some of these families at the ratio of 1 supernodulation : 2 hypernodulation : 1 non-nodulation. In other words, two categories of the enhanced nodulation phenotypes were observed (Figure 7.2). The homozygous supernodulating plants had the characteristic nts382 pattern of nodulation, wherein large numbers of nodules completely covered the entire tap and lateral roots. In contrast, the hypernodulating plants had a lower number of nodules compared to the supernodulators and the nodules were randomly distributed on the root system at a lower density (Figure 7.4). The non-nodulation phenotype carried the supernodulation allele *(nts)* in a homozygous condition and was classified as the double recessive mutant. It formed 25 per cent of the supernodulation F_3 segregation progeny. The observed F_3 segregation of the F_2 enhanced nodulation plants gave a good fit to the observed values when tested by Chi-square analysis (Table 7.3), and indicated that (i) the homozygous double mutant was non-nodulating, (ii) plants

homozygous wild type for the non-nodulation locus but homozygous for nts382 were supernodulating, and (iii) plants heterozygous for the non-nodulation locus and homozygous for the *nts*382 allele were hypernodulating. The homozygous F_3 supernodulation plants produced only true breeding supernodulating plants whereas the hypernodulating F_3 plants segregated in the F_4 into three classes of supernodulation, hypernodulation and non-nodulation in the ratio of 1 : 2 : 1. Again Chi square analysis gave a good fit to the expected values. The double recessives carrying the recessive alleles in a homozygous condition were pure breeding.

Plant growth studies of the non-nodulating progeny obtained in the F_3 segregation of the cross between nts382 and the non-nodulation mutants indicated that the root lengths of the non-nodulating plants (double mutants) were larger than the supernodulation and hypernodulation segregants when grown on nitrate.

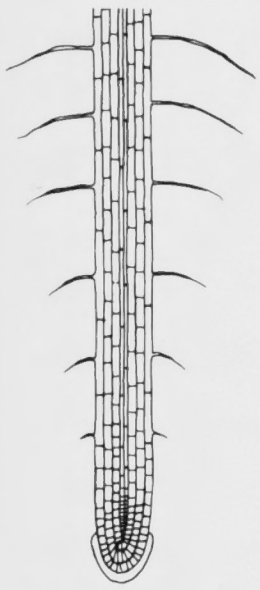
In summary, in a supernodulation plant when the non-nodulating locus is homozygous wild type, the resulting progeny are all supernodulating. However, when the non-nodulating locus is heterozygous, the progenies produce a lesser number of nodules and are hypernodulating. It thus appears that there is a dose effect of the non-nodulating allele on the expression of supernodulation. Developing this line of argument further, these observations suggest that *nod*49, *nod*139 and *nod*772 mutations are incompletely dominant but this nature of inheritance is masked by autoregulation *(aut)* in the wild type. Figure 7.10 presents a model of the interaction of the supernodulation mutant with the non-nodulation mutants. Here *aut* refers to the autoregulation allele while *nod* refers to the non-nodulation allele. In this model, in the wild-type parent Bragg ($aut^{+/+}nod^{+/+}$), most infections (sub-epidermal cell divisions) do not lead to the formation of infections as the early formed nodules prevent subsequent infections from forming nodules due to the autoregulation mechanism. However, in the supernodulation mutant *nts382* ($aut^{-/-}nod^{+/+}$), many more infection events (sub-epidermal cell divisions) lead to the formation of nodules as autoregulation is defective. In the non-nodulating mutant ($aut^{+/+}nod^{-/-}$), there are very few actual infections and some pseudoinfections (Chapter 5). Occasional nodules are

Figure 7.10 **Demonstration of incomplete dominance in nod49, nod139 and nod772 requires the absence of autoregulation.** A model explaining incomplete dominance of the non-nodulating mutants at the morphological level and its recessive nature in the autoregulated background.

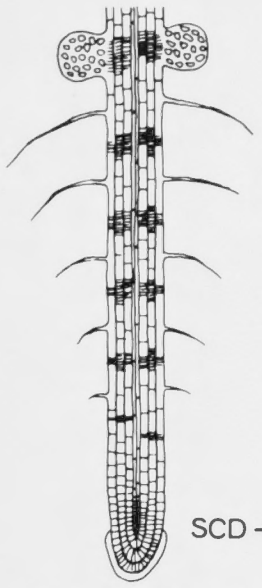
SCD - sub-epidermal cell division

aut - autoregulation allele

nod - non-nodulation allele

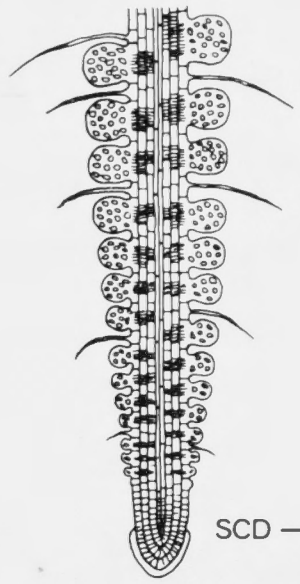


NON-NODULATION
(aut $^{+/+}$ nod $^{-/-}$)



SCD ⟶✕ NODULES

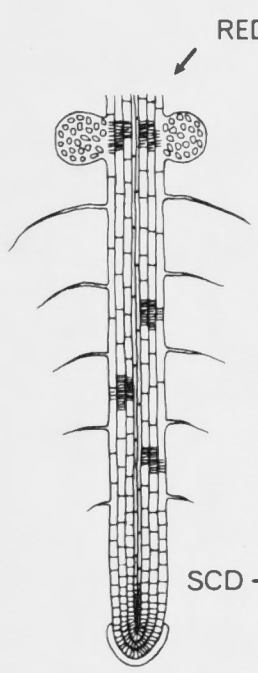
NORMAL NODULATION
(autoregulation)
aut $^{+/+}$ nod $^{+/+}$



SCD ⟶ NODULES

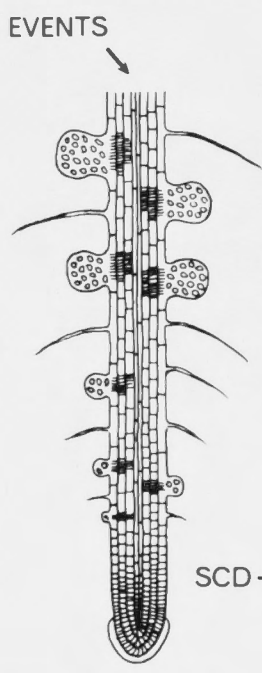
SUPERNODULATION
(no autoregulation)
aut $^{-/-}$ nod $^{+/+}$

REDUCED INFECTION EVENTS



SCD ⟶✕ NODULES

HETEROZYGOUS NON-NODULATION
(autoregulation)
aut $^{+/+}$ nod $^{+/-}$



SCD ⟶ NODULES

HETEROZYGOUS NON-NODULATION
(no autoregulation)
aut $^{-/-}$ nod $^{+/-}$

formed from these occasional infections. It is proposed that there are reduced infection events (sub-epidermal cell divisions) in the non-nodulation heterozygote *(aut*$^{+/+}$*nod*$^{+/-}$*)* with wild type but due to autoregulation, the number of nodules formed are of the wild-type level as seen in Figure 7.10. On the other hand, in the non-nodulation heterozygote with the supernodulation mutant (*aut*$^{-/-}$*nod*$^{+/-}$*)* there is a reduced number of infection events and since there is no autoregulation most infections result in nodules leading to the hypernodulation level (Figure 7.10). Clearly, autoregulation may limit the detection of mutants defective in nodulation (i.e. the formation of sub-epidermal cell divisions) and leaky mutants will not be detected. However, leaky mutants altered in this early developmental stage can be more easily detected in a supernodulation background lacking autoregulation. This is important to both plant and bacterial geneticists. Mutations may occur in nodulation genes and result in less infections but leaky defects will be masked by autoregulation. Therefore, in order to visualize the effect of mutation on nodulation genes (plant and bacterial) needed for nodule initiation (i.e. sub-epidermal cell division), leaky mutants should be checked in a background that lacks autoregulation. The approach used here indicates that nod49 is most likely to be incompletely dominant at the morphological level but behaves as a recessive in the autoregulated background. With the existence of autoregulation only null mutants will be reliably detected.

Supernodulation on the Bragg roots confirmed both the double recessive mutant and that non-nodulation is epistatic over supernodulation, as only shoots with the homozygous recessive *aut* genotype can produce supernodulation on the Bragg rootstocks (Chapter 6). The double mutant graft on the Bragg rootstock produced a reduced root system similar to the nts382 control grafts confirming that supernodulation results in decreased root growth whereas the Bragg control grafts had a significantly longer root system.

The double mutant DM49 (nts382 x nod49) was compared for its growth characteristics to both its parents nts382 and nod49 and the cv. Bragg from which all these mutants were originally isolated. The data presented indicate that when the mutants were totally dependent on nitrate as their sole nitrogen source, the root and shoot lengths of all the mutants were similar. The root, shoot and

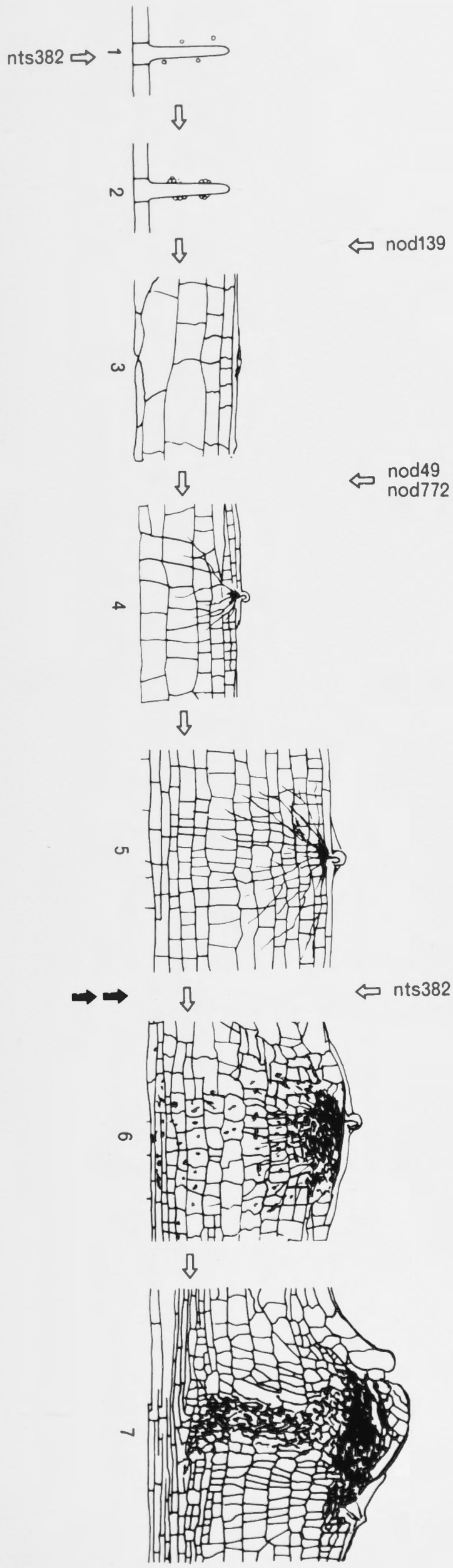
plant dry weight of DM49 were similar to those of nod49 and Bragg, confirming that these mutants are not altered in the assimilation of nitrate. All the mutants including nts382 had similar plant growth characteristics for the first two weeks after germination. This could be the result of similar nitrogen and protein content in the seeds of the mutants (Table 3.1). When the mutants were dependent on symbiotically fixed nitrogen, the root length, and root, shoot and plant dry weights of DM49 and nod49 were similar for two weeks. After 4 weeks, however, the plant growth parameters of both were considerably smaller than Bragg due to their inability to nodulate and fix nitrogen. Mutant nts382 was significantly lower than all the other lines in these characteristics. These results confirm the earlier reports that supernodulation in nts382 leads to a reduction in growth rate and plant size (Carroll *et al.*, 1985b; Day *et al.*, 1986). Addition of nitrate and inoculation of the mutants resulted in a similar root length of DM49, nod49 and Bragg but nts382 had a significantly shorter root length. The shoot, root and plant dry weights of nod49 was similar to Bragg. Mutant DM49 resembles the non-nodulating parent nod49 more closely in its growth characteristics than the supernodulation parent nts382 but when inoculated and grown on nitrate it was smaller than Bragg and nod49 control after 4 weeks of growth. This indicates that the expression of supernodulation *per se* may not be the sole cause of decreased growth in nts382. As described in Chapter 5, nts382 has a greater number of *B. japonicum* in the rhizosphere particularly after 17 days of growth. Mutant DM49 may also have an increased number of *B. japonicum* in the rhizosphere which may contribute to its poorer growth when inoculated and grown on nitrate. This aspect needs to be confirmed. Should this be the case, then an increased number of *Bradyrhizobium* in the rhizosphere could probably act synergistically with supernodulation *per se* to decrease plant growth in nts382. This is an important aspect since the relationship between supernodulation itself and poor growth may be separated and this would be important for the agronomic use of supernodulation.

The non-nodulation mutants nod49 and nod772 are blocked at the early infection stage of nodulation, namely at the stage of cortical cell division. Sub-epidermal cell divisions in these mutants are rare and are restricted to the stage I pseudoinfections (divisions not associated with infection threads) while nod139 does not have any form of sub-epidermal cell divisions (Chapter 5). The

supernodulation mutant nts382 is altered in its autoregulation mechanism resulting in an unrestricted number of nodules on the root system. It appears that most of the infections in nts382 are successful in the formation of nodules when compared to the wild type. The double mutants (DM49, DM772 and DM139) of the crosses between nts382 and nod49, nod772 and nod139 have a non-nodulation phenotype. Here, the non-nodulation gene in these mutants epistatically suppresses the supernodulation gene. This mechanism can be explained in terms of the nodule development mechanism as illustrated in Figure 7.11, and is an example of developmental epistasis. Here, the alteration in the autoregulatory response in nts382 is masked by the blockage of the non-nodulation mutants at the sub-epidermal cell division stage, thereby resulting in a non-nodulation phenotype in the double mutants.

Figure 7.11 **Site of alteration in nodulation in nod49, nod772, nod139 and nts382.** The vertical arrows indicate the main steps leading to nitrogen fixation in most legumes. The horizontal arrows indicate the site of alteration in the mutants and the double arrow indicates the autoregulation stage in the wild type. The alteration in the autoregulation response in nts382 is masked by the blockage of the non-nodulation mutants at the sub-epidermal cell division (scd) stage, resulting in a non-nodulation phenotype in the double recessive mutant. N.B: The non-nodulation mutants nod49 and nod772 have a considerably lowered number of sub-epidermal cell divisions and these divisions rarely progress beyong stage III.

1- Root colonization
2 - Root attachment
3 - Stage I of Scd
4 - Stage III of Scd
5 - Stage IV of Scd
6 - Stage VI of Scd
7 - Stage VIII of Scd


nts382
1
2
nod139
3
nod49
nod772
4
5
nts382
6
7

CHAPTER 8

GENERAL DISCUSSION

A basic and complete knowledge of the mechanisms and functions of the various stages of the legume-rhizobia symbiosis is essential for optimizing the benefits of any agronomic improvements of the nitrogen fixation capacity in legumes. The nodule development sequence in soybean, for example, has been extensively researched but despite this it is not yet well understood. Also knowledge of the genetics of the legume and the microsymbiont offers a correlation between the causalities connecting function (i.e. phenotype) to a macromolecular change (either DNA or gene product). Hence the isolation and characterization of non-nodulation, ineffective nodulation, delayed nodulation, altered host range mutants and supernodulation mutants in both the legume and the microsymbiont can enable the fragmentation of this complex multi-step process and enhance the understanding of nodule ontogeny.

In 1954, Williams and Lynch first described a naturally-occurring non-nodulation soybean which carried a spontaneous mutation at the allele denoted as rj_1. Despite thirty years since the discovery of this non-nodulation mutant and the identification of other non-nodulation mutants in other legume species, the nature of the non-nodulation phenomenon and the exact stage of arrest in symbiotic development has not been determined. In this thesis, three induced non-nodulation mutants of soybean nod49, nod139 and nod772, a supernodulation and nitrate tolerant symbiosis mutant nts382 (Carroll *et al.*, 1985 a,b; 1986) along with the naturally-occurring variant rj_1 (Lee)(Williams and Lynch, 1954) were characterized in more detail and the stage(s) of alteration in nodulation in these mutants were identified.

The non-nodulation mutants are not altered in the assimilation of nitrogen and carbon when grown on nitrate. Hence, the aberration in the non-nodulation mutants is specific to the nodulation process. This was substantiated by the finding that these mutants express constitutive nitrate reductase (cNR) and inducible nitrate reductase (iNR) activity similar to the wild-type Bragg. These enzymes are not directly related to nodulation. Weber (1966) also observed that rj_1 plants had higher seed and dry matter yields when grown on increased nitrogen in the field. The supernodulation mutant nts382 is also unaltered in the

assimilation of nitrogen and carbon reiterating the results obtained for nts382 by Carroll *et al.* (1985 a,b) and Day *et al.* (1986).

A strain and inoculant titre dependent occasional nodulation phenotype was observed on the non-nodulation roots when screened for nodulation with several *Bradyrhizobium, Rhizobium fredii, Rhizobium* sp. and *Parasponia Bradyrhizobium* strains in Leonard jars. DuTeau *et al.* (1986) observed a few small effective nodules on lateral roots of several rj_1 plants when inoculated with *Rhizobium fredii* strains USDA191 and USDA205 at 1 x 10^8 cells. ml^{-1}. La Favre and Eaglesham (1984) obtained a similar result but improved the nodulation at higher inoculant doses with the rj_1 mutant. High inoculant dose causes occasional nodules to be formed on the other non-nodulation soybean mutants. These nodules were often quite large and located on the lateral roots and root tissue that developed later in plant growth denoting a delayed nodulation pattern. They were, however, similar to the wild-type nodules in morphology, development and acetylene reduction activities. The possibility that high cell numbers of rhizobia are required to provide either adequate cells capable of infection or certain bacterial by-product(s) may explain the occasional nodulation of these mutants (La Favre and Eaglesham, 1984). The non-nodulation mutants do not nodulate in the field (Clark, 1957; D. Herridge, *pers. comm.*, New South Wales, Australia; E. Appelbaum, *pers. comm.*, Wisconsin, USA). This indicates that sub-optimal inoculant levels and/or increased microbial contamination may decrease the bacterial signals which are required for the induction of sub-epidermal cell divisions and nodulation of these lines. The strain specific occasional nodulation of the mutant rj_1 was originally correlated with the ability of the *B. japonicum* strains to produce rhizobitoxine. Devine and Weber (1977) and Devine *et al.* (1983a) postulated the requirement of a high threshold of rhizobitoxine for the infection of the mutant rj_1. This hypothesis was disproved by La Favre and Eaglesham (1984) who found no correlation between the ability of a rhizobial strain to nodulate rj_1 plants and produce rhizobitoxine. The number of nodules and nodule dry weight of mutant nts382 increased significantly when it was inoculated with *B. japonicum* strain CB1795 at a medium inoculant (1 x 10^7 viable cells. ml^{-1}) and high inoculant

(1×10^9 viable cells $.ml^{-1}$) inoculant indicating an increased nodulation response in nts382 compared to no significant differences in these parameters when Bragg was inoculated at different inoculant titres (Chapter 4, Table 4.1).

Several attempts were made to suppress the non-nodulation phenotype but without success (Chapter 4). Removal of cotyledons from one week old seedlings (thereby excluding any potentially inhibitory factors present in them which deter nodulation) did not help to circumvent non-nodulation. Tanner and Anderson (1963) demonstrated using the grafting technique that the rj_1 mutant did not lack a cotyledonary factor necessary for nodulation nor did it have a cotyledonary factor that inhibited nodulation. The same was demonstrated for nod49, nod772 and nod139 (Chapter 4). Perhaps the cotyledons are not required for nodulation since Gresshoff (1980) observed in tissue culture organogenesis from protoplast-derived clones that white clover plants nodulate without cotyledons. Cotyledonary reserves are, however, essential for plant growth during early seedling stage. The notion that these mutants may be slow to nodulate and that occasional nodulation is seen on the root tissue which developed late in plant growth were tested by vegetatively propagating the seedlings. This approach did not yield any positive results. Further, the addition of small amounts of nitrate to the plant nutrient solution and the strategy of exposing the roots to high nitrate for the initial two weeks followed by a period of nitrate deprivation and subsequent inoculation with a high dose of *Bradyrhizobium japonicum* did not help to suppress the non-nodulation phenotype (Chapter 4).

The non-nodulation characters *nod*49, *nod*139 and *nod*772 and the supernodulation trait *nts*382 are inherited as Mendelian monogenic recessives as seen in crosses to the parent cv. Bragg. This was also demonstrated with the cross of *nod*49 and *nod*772 to the cv. Clark. The monogenic inheritance of the non-nodulation mutants was confirmed in the F_2 and also in the F_3 progenies thus discounting the possibility of multigenic inheritance. Williams and Lynch (1954) reported that the rj_1 mutation is inherited as a monogenic recessive. On the other hand, the ineffective nodulation in Rj_2 (Caldwell, 1966), Rj_3 (Vest, 1970) and Rj_4 (Vest and Caldwell, 1972) are inherited as

monogenic dominant loci. Mutants nod49 and nod772 are allelic to either rj_1 as determined by complementation tests. In contrast, nod139 is not allelic to rj_1, *nod*49 or *nod*772 and is defective in a separate function required for nodulation. The gene symbol rj_6 is proposed for this new gene conditioning non-nodulation in soybean with reservations due to the inappropriateness of the nomenclature (Chapter 3). The supernodulation character in nts382 is determined by a locus separate from rj_1 and rj_6. Mutants nod49 and nod772 have different phenotypic characteristics indicating that they came from independent mutations (Chapter 4; Mathews *et al.*, 1987a). However, they are allelic to rj_1 which suggests either a 'hotspot' for mutation at the rj_1 locus or a physically close aggregation in such a manner that complementation does not occur between the mutations. The existence of 'hotspots' for mutation at the *sym*-5 locus in peas has been demonstrated by Kneen and LaRue (1984b). In plant disease interactions, the existence of physically close genes has been reported in barley chromosome 5 and wheat chromosome 2 where genes conferring resistance to several diseases appear to be physically close (Day *et al.* 1983). The existence of nodulation neighbourhoods within the genome of some legumes similar to the organization of the leghemoglobin gene is possible.The linkage of *nod*139 (rj_6) to rj_1 and the linkage of rj_6 to both *nod*49 and *nod*772 was not clearly discernible due to the lack of any known morphological markers in these mutants. The F_2 progeny segregation when tested for goodness-of-fit to the expected ratio fitted into both the tight linkage and no linkage hypothesis.

The non-nodulation mutants of soybean had similar root exudates to the parent Bragg on a biological level as indicated by the co-culture and nodulation efficiency studies. In comparison to Bragg there were no inhibitory substances detectable from the non-nodulating mutants and no crossfeeding of missing substances from either the wild type or supernodulation mutant. The reduction in nodulation of the supernodulation plants by co-culture with either Bragg or the non-nodulation mutants may be explained either in terms of competition of the wild-type Bragg or the non-nodulation plants with the smaller supernodulation plants for plant nutrients. However, this possibility could be ruled out since the

plant dry weights of nts382 remain unaltered irrespective of the companion plant in the co-culture. The possible production of an inhibitor which might be absent in nts382 needs to be investigated further. This could be clarified using the double recessive mutants (DM49, DM772 and DM139) obtained from crosses between nts382 and the non-nodulation mutants. The double recessive mutants are non-nodulating, yet possess a supernodulation shoot (Chapter 7) and would not be expected to have the presumptive inhibitor. Likewise, the intermediate supernodulating mutant nts1116 could also be used in the clarification of this point. However, it is interesting to note that nts382 supported a larger number of *B. japonicum* strain CB1809 in the rhizosphere. This may also be indicative of alterations in the nts382 root exudate.

Inoculation of precultured *Bradyrhizobium japonicum* strain USDA110 in the non-nodulation mutant exudates showed neither an increase nor a decrease in nodulation of the Bragg roots. These results are in contrast to that of Elkan (1961) who reported that the rj_1 plants excreted an inhibitory compound when tested for its ability to affect nodulation in co-culture with the nearby isogenic Rj_1 (nodulating) plants. However, Eskew and Schrader (1977) were also unable to repeat Elkan's observations as was the case in this study (Chapter 4). Electrophoretic analysis of root exudates of nod49, nts382 and Bragg indicated a similar protein pattern in all these genotypes on both one and two dimensional gels (Mathews *et al.,* in preparation). Similarly, radioactively labelled (^{35}S) root exudates when subjected to immunoprecipitation with the antibody to soybean lectin (SBL) showed similar amounts in nod49, nts382 and Bragg (Mathews *et al.*, in preparation). Furthermore, methanol/water extracts from uninoculated seedling roots were able to induce *nod* C-*lac* Z fusions of USDA123 (pEA23-2) giving similar ß-galactosidase activities in all cases. This confirmed that nod49, nts382 and Bragg possess a similar distribution of stimulatory and inhibitory substances (Kosslak *et al.,* 1987) capable of affecting the expression of the conserved nodulation genes in the microsymbiont (Mathews *et al.,* in preparation). Even though the non-nodulation mutants produce compounds that induce the expression of the conserved *nod* genes in *Bradyrhizobium*, they do not respond to the ontogenic consequence of *nod* gene expression and do not produce sub-epidermal cell divisions.

The non-nodulation mutants are similar in the rhizosphere colonization of *B. japonicum* strain CB1809 denoting that they are not blocked at this stage of nodulation. Mutant nts382 and the intermediate supernodulating mutant nts1116 supported a considerably larger population of *B. japonicum* strain CB1809 in the rhizosphere than either the wild-type Bragg or the non-nodulation mutants. There were no differences in the attachment of *B. japonicum* USDA1-110ARS to the nodulating and non-nodulating roots of the genotypes when tested for attachment using *B. japonicum* strain USDA1-110 ARS. All the non-nodulating mutants attached as well as the supernodulation and the wild-type roots. Similarly, preincubation of *B. japonicum* USDA110 in either the non-nodulation mutant exudate or the exudate of the wild type confirmed that the non-nodulation mutants are not different and that they do not have any inhibitory or stimulatory substances which may either decrease or increase or even supplement a lack of some substance in the root exudates of these mutants. Clearly, the blockage in the non-nodulation mutants is subsequent to root colonization, induction of conserved inducible *nod* genes and attachment of the microsymbiont to the roots (Chapter 1, Table 1.1)

Cytological examination of the roots of these genotypes revealed a dose dependent and strain dependent nodule initiation response since a higher number of sub-epidermal cell divisions was found on the non-nodulation mutants when inoculated with *B. japonicum* strain CB1795 compared to USDA110. The high inoculant titre (1×10^9 viable cells. ml^{-1}) initiated a higher number of sub-epidermal cell divisions compared to the low inoculant titre (1×10^5 viable cells. ml^{-1}). The non-nodulation mutants had few sub-epidermal cell divisions which were mainly ontogenetically retarded pseudoinfections. The mutants appear to be blocked at this early step of nodule initiation and may require a higher concentration of either some bacterial signal or by-product or both to induce divisions in the outer cortex of the root. Any nodulation on these mutants requires a high cell titre of rhizobia and occasional nodules are often observed where rhizobia have a better chance to aggregate and produce either signals or by-products perhaps at the cotton plug region of a Leonard jar.

Mutant nod139(rj_6) did not have any sub-epidermal cell divisions indicating that it is blocked a little earlier in the nodulation process. Mutant nod772 is a leaky mutant as it has some curled root hairs (Table 4.2, Chapter 4) and it also has a few sub-epidermal cell divisions even at low inoculation of *B. japonicum* strain CB1795 (1×10^5 viable cells. ml^{-1}) (Table 5.12, Chapter 5). It also produced a few more nodules compared to the other non-nodulation mutants. The wild-type Bragg regulates sub-epidermal cell division at stage IV and autoregulation acts at this stage since infection events are reduced subsequent to this stage. The supernodulation mutant nts382 is altered in the autoregulation step since more infections in this mutant lead to nodules resulting in supernodulation. The phenomenon of autoregulation, wherein the plant has a rapid regulatory mechanism to control the number of nodules on its root system (Pierce and Bauer, 1983; Calvert *et al.*, 1984), is altered in nts382.

Mutant nts382 nodulates quicker than Bragg (B.J. Carroll, P.M. Gresshoff and A.H. Gibson, *pers. comm.*). It has a higher number of advanced stages of sub-epidermal cell divisions compared to the wild-type Bragg which is slowed at stage IV onwards. It is therefore possible that autoregulation is a phenomenon of the immature zone of the root and is manifested as the rate of divisions since in Bragg the number of infection events and nodules are drastically reduced below the RT (root tip) mark made at the time of inoculation of the root. The rate of infection in nts382 is developmentally more rapid than in Bragg since many more sub-epidermal cell divisions go quickly through to the advanced stages of sub-epidermal cell division. Mutant nts382 also has more nodules below the RT mark compared to Bragg (Chapter 5).

Sub-epidermal cell division and infection (markedly curled root hairs associated with infection threads) stages are defective in the non-nodulation mutants which indicates that these two processes could be regulated in a coordinated manner in the wild type. However, if different signals induce these two events, then the coordinate acceptance of these signals must exist. Alternatively, sub-epidermal cell divisions could occur first and may be required for marked root hair curling and infection formation. This can be clarified by the isolation of rhizobia and/or plant mutants blocked at either of these two stages.

The fact that pseudoinfections exist suggests that sub-epidermal cell divisions do not require infection thread formation and that there are mutants of rhizobia that produce sub-epidermal cell divisions without infection thread formation. The simplest explanation of these observations is that sub-epidermal cell division is required for infection thread formation.

Pseudoinfections (sub-epidermal cell divisions not associated with an infection thread) do not progress beyond stage III in the wild-type Williams and were found less frequently in the younger region of the root , i.e. below the shortest emerging root hairs (Calvert *et al.*, 1984). Similar results were obtained with the wild-type Bragg (Chapter 5). In nts382, however, pseudoinfections were frequently observed to progress to stage V and were numerous below the RT mark made at the time of inoculation. This indicates that nts382 is different from the wild type in the lack of cell division regulation in pseudoinfections, and that in the wild type autoregulation slows down both pseudoinfections and infections. In nts382 which lacks autoregulation, infections are not slowed down below the RT and they are generally more advanced. The presence of an infection thread must enhance the rate of cell division since pseudoinfections in both Bragg and nts382 do not reach the advanced stage where it can be detected by the naked eye as a protuberance on the root.

The absence of markedly curled root hairs in the nod49, nod139 and rj_1 when inoculated with *B. japonicum* strain USDA110 at medium (1×10^8 viable cells. ml^{-1}) cell numbers indicated a blockage in this early step of infection. The leaky nature of the non-nodulation mutant nod772 was correlated to a leakiness at the root hair curling stage, i.e. the presence of a small per cent of marked curled root hairs. An erratic distribution of root hairs on the nod139 roots indicates another developmental alteration in this mutant which may be associated with the non-nodulation mutation but this requires confirmation. Mutant nts382 had a similar percentage of curled root hairs to Bragg a week after inoculation and a slightly higher number of curled hairs two weeks after inoculation. This indicates that supernodulation is not the result of increased infection, instead an increased ability of infections to be translated into nodules. A summary of the phenotypic characteristics of the soybean mutants is outlined in Table 8.1.

Table 8.1 **Summary of the phenotypes of the soybean mutants at different stages of the symbiotic process.** The mutants are described relative to the wild-type Bragg.

Table 8.1

Symbiotic stage	Mutant	Phenotypic description[a]	Reference
Colonization	nts382;	enhanced	Table 5.7
	nod49, nod772 and nod139	normal	Table 5.7
Root exudate			
(i) Lectin	nts382;	normal	Mathews *et al.* (in prep.)
	nod49	normal	"
(ii) *nod* gene induction	nts382;	normal	"
	nod49	normal	"
Attachment	nts382;	normal	Tables 5.8 and 5.9
	nod49, nod772 and nod139	normal	Tables 5.8 and 5.9
Sub-epidermal cell divisions	nts382;	slightly enhanced	Table 5.11
	nod49,nod772;	severely reduced	
	nod139	absent	Table 5.11
Autoregulation	nts382;	absent	Figures 5.2 and 5.3
	nod49, nod772 and nod139	normal in shoot	Tables 6.1, 6.2 and 6.3
Nodule development and function	nts382;	normal	Day *et al.* (1987)
	nod49, nod772 and nod139	normal	Table 4.1; Figure 4.2

[a] relative to wild-type Bragg

The normal infection mechanism involving curled root hairs and infection threads was observed in the occasional nodule formed on the non-nodulation mutant. Infection threads in the non-nodulation mutants were observed in the root hairs (Chapter 5). Pueppke and Payne (1987) observed the presence of infection threads in occasional nodules on *rj*$_1$ and confirmed this observation by transmission electron microscopy. Nodules on the induced non-nodulation mutants were often associated with a lateral root emergence point, in which case the presence of infection thread in the nodule was difficult to observe by light microscopy.

The tissue control of non-nodulation in all the non-nodulation mutants of soybean resides in the root, and factor(s) determining resistance to nodulation are not systemic (Chapter 7). In other words, irrespective of whatever was grafted as the scion, the root of a non-nodulation mutant always remained non-nodulated. However, the adventitous roots arising from the scions of nodulating genotypes were always nodulated. This indicated an absence of translocation of an inhibitory substance(s) from the rootstock of the non-nodulation mutant to the shoots of the nodulating scion, thereby preventing nodulation of the adventitous roots from the scion. In other words, the factor(s) conditioning non-nodulation are localized in the roots of the non-nodulation mutant. In contrast, supernodulation in nts382 is controlled by the shoot as the scion of nts382 can induce supernodulation on the wild-type root stock. Grafting the non-nodulation mutants on the supernodulation rootstock resulted in a wild-type phenotype showing that no interaction occurred. Normal autoregulation response was observed when the Bragg shoots were grafted on nts382 roots indicating that the supernodulator is not altered in the root factors which affect autoregulation. Supernodulation was also induced on both the root stock of the intermediate supernodulator nts1116 and the cv. Williams by nts382 (Delves *et al.*, 1986) and other supernodulation mutants (Delves *et al.*, 1987). The non-nodulation shoots displayed the normal autoregulation response (Chapter 6).

The non-nodulation character in mutants nod49, nod772 and nod139 is epistatic on supernodulation in mutant nts382 as seen in the crosses of the

supernodulation mutant to these non-nodulation mutants. The blockage in these two non-nodulation complementation groups is realized much earlier in the nodulation process than the defect in nts382.

The interactions of the supernodulation locus *nts*382 and the non-nodulation loci *nod*49, *nod*772 and *nod* 139 at a genetic level indicate that these characters are controlled by separate loci as seen in the wild-type pattern of nodulation in the F_1 progeny. Epistatic gene interaction and the unlinked nature of the supernodulation and non-nodulation genes were observed when the F_2 progeny segregated into three phenotypic classes of 9 wild type : 3 supernodulation : 4 non-nodulation categories. Twenty five per cent of the non-nodulation category were the double mutants which were epistatically placed within the non-nodulation group. Therefore, this substantiates the fact that the non-nodulation root suppresses supernodulation.

Some interaction was observed between the supernodulation and the non-nodulation complementation groups. The hypernodulation plants (i.e. plants heterozygous for the non-nodulation locus and homozygous for the supernodulation allele), obtained from the F_2 segregants with enhanced nodulation, were distinguished from the homozygous supernodulation plants by the lowered nodule number and sparse distribution of nodules on the root. Furthermore, nodules were present at a lower density compared to the homozygous supernodulation plants in which nodules completely covered tap and lateral roots. All the progeny bred true when the non-nodulation locus was homozygous wild type whereas heterozygosity at the non-nodulation locus resulted in hypernodulating phenotype, indicating a dose effect of the non-nodulation allele on the expression of supernodulation. Perhaps, mutant alleles *nod*49, *nod*772 and *nod*139 are incompletely dominant and are masked by autoregulation with gene *nts*382 in a homozygous background. Alternatively, a rate hypothesis could apply here. In Bragg, most infections are restricted at stage IV whereas in nts382 infections quickly progress to the advanced stages of sub-epidermal cell divisions. The fact that the non-nodulation pseudoinfections were at stage I and II indicates that the rate of sub-epidermal cell division development is slower in the non-nodulation mutants. To distinguish between

the two the heterozygous non-nodulation plants should be observed for sub-epidermal cell divisions.

The homozygous F_3 plants produced only true breeding progeny whereas the hypernodulating plants segregated further into three classes of supernodulation, hypernodulation and non-nodulation. The non-nodulation phenotype carried the supernodulation allele in a homozygous condition and was verified by grafting onto Bragg root stocks as only shoots with homozygous recessive supernodulation genotype can produce supernodulation on the Bragg root stock. The growth characteristics of the double mutant of nts382 x nod49 indicate that it is more similar to the non-nodulation parent nod49. The small plant size of the double mutant compared to the wild-type Bragg and nod49 control indicates that the supernodulation phenotype *per se* may not be the sole cause of decreased growth in nts382. The enhanced colonization may also contribute to this effect (Chapter 7).

Future prospectives

This study has generated several areas of future lines of work which will facilitate the understanding of symbiotic nitrogen fixation in soybean.

The non-nodulation mutants can be used in the estimation of nitrogen fixation in isogenic wild-type soybeans. This requires recurrent backcrossing and selection to purify the genetic background of these mutants. These mutants could also be used in screening for strains of the microsymbiont that can suppress the non-nodulation in these mutants and help overcome the problem of competition of genetically inferior indigenous strains in the soil which compete with superior inoculant strains (Moawad *et al.*, 1984).

The non-nodulation mutants can be used in the study of the various steps involved in nodule initiation and formation. The location of tissues in the control of non-nodulation (root) and supernodulation (shoot) can be used for a detailed mechanistic analysis of nodulation aberration in these mutants. The use of grafting techniques together with biochemical techniques will enable the

identification of the factor(s) involved in non-nodulation and supernodulation which in turn will elucidate the mechanisms and regulation of nodulation in the wild-type soybean. The biochemical basis of mutations in the mutants may be detected by an analysis of *in vitro m* RNA translation products from inoculated and uninoculated non-nodulation mutants. This could help to estimate the background levels of root proteins and shoot proteins which are specific to infection that may be missing in the roots and shoots of the mutants, thereby demonstrating their importance to wild-type nodulation. Plant gene products involved in the early stages of nodule initiation can be identified by the use of the non-nodulation mutants. Since these mutants are blocked at the sub-epidermal cell division stage of nodulation, they can serve to answer questions regarding the synthesis of signals for sub-epidermal cell divisions, the role of the legume in the production of plant signals in early infection and its response to signals emanating from the microsymbiont.

Crossing *nod*139 (rj_6) with the ineffective nodulation (Rj_2 and Rj_4) genes will help to determine whether rj_6 is in a different complementation group in relation to these genes and will also enable a gene symbol to be assigned to the recessive gene tentatively designated as rj_6 which conditions non-nodulation in *nod*139. However, the distinct phenotype of nod139 compared to these other variants suggests that it is indeed a new gene affecting nodulation. Gene Rj_3 which conditions ineffective nodulation no longer exists (T. E. Devine *pers. comm.*) and hence is not available for further studies.

The double recessive mutant obtained from the crosses between the supernodulation and non-nodulation mutants has a non-nodulation phenotype and can be used successfully to study the supernodulation allele in a plant devoid of nodules yet still exposed to the microsymbiont in the rhizosphere. This eliminates the complexity of the supernodulation phenotype associated with the supernodulation mutant.

Finally, this study has indicated the limitations imposed by autoregulation in the detection of mutants altered in sub-epidermal cell divisions since leaky mutants altered in the early developmental stages (such as sub-epidermal cell divisions) will go undetected in the autoregulated backgound and can be detected more readily in a supernodulation background which lacks autoregulation. This approach of isolating mutants altered in nodulation can be applied to both the plant and the microsymbiont.

BIBLIOGRAPHY

Allen, E.K. and Allen, O.N. (1958) Biological aspects of symbiotic nitrogen fixation. In: W. Ruhland, ed., Encyclopedia of Plant Physiology. Vol. VIII, Berlin-Heidelberg-New York, Springer, pp. 48-118.

Allen, O.N. and Allen, E.K. (1940) Response of the peanut plant to inoculation with *Rhizobia*, with special reference to morphological development of the nodules. *Bot. Gaz.* 102, 121.

Alvey, N., Banfield, C., Baxter, R., Gower, J., Krzanowski, W., Lane, P., Leech, P., Nelder, J., Payne, R., Phelps, K., Rogers, C., Ross, G., Simpson, H., Tood, A., Wedderburn, R. and Wilkinson, G. (1977) GENSTAT - A general statistical program. Lawes Agricultural Trust, Rothamsted Experimental Station.

Ames, P. and Bergmen, K. (1981) Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. *J. Bact.* 148, 728-729.

Anolles, G. C. and Favelukes, G. (1986) Quantitation of adsorption of rhizobia in low numbers to small legume roots. *Appl. Environ. Microbiol.* 52, 371-376.

Arora, N. (1954) Morphological development of the root and stem nodules of *Aeschynomene indica* L. *Phytomorphology* 4, 211-216.

Atkins, C.A. (1986) The legume/*Rhizobium* symbiosis. Limitations in maximizing nitrogen fixation. *Outlook Agriculture* 15, 128-134.

Atwood, S. S. and Sullivan, J. T. (1943) Inheritance of a cyanogenetic glucoside and its hydrolyzing enzyme in *Trifolium repens*. *J. Heredity* 34, 311-320.

Aulie, R.P. (1970) Boussingault and the nitrogen cycle. *Proc. Am. Philos. Soc.* 114, 435-479.

Avers, C.J. (1984) Mendelian inheritance. In: Genetics. Willard Grant Press, Boston, pp 24-27.

Badenoch-Jones, J., Flanders, D.J. and Rolfe, B.G. (1985) Association of *Rhizobium* strains with roots of *Trifolium repens*. *Appl. Environ. Microbiol.* 49, 1511-1520.

Basset, B., Goodman, R.N. and Novacky, A. (1977) Ultrastructure of soybean nodules. I: release of rhizobia from the infection thread. *Can. J. Microbiol.* 23, 573-582.

Bauer, W. D. (1981) Infection of legumes by rhizobia. *Ann. Rev. Plant Physiol.* 32, 407-449.

Bauer, W. D., Bhuvaneswari, T. V., Calvert, H. E., Law, I. J., Malik, N. S. A. and Vesper, S. J. (1985) Recognition and infection by slow-growing rhizobia. In: H. J. Evans, P. J. Bottomley and W. E. Newton, eds., Nitrogen Fixation Research Progress. Maritnus Nijhoff Publ., Dordrecht, The Netherlands, pp. 247-253.

Bender, G.L., Nayudu, M., Goydych, W. and Rolfe, B.G. (1987) Early infection events in the nodulation of the non-legume *Parasponia andersonii* by *Bradyrhizobium*. *Plant Sci.* 51, 285-293.

Bergersen, F.J. (1961) The growth of *Rhizobium* in synthetic media. *Aust. J. Biol. Sci.* 14, 349-360.

Bergersen, F. J. and Nutman, P. S. (1957) Symbiotic effectiveness in nodulated red clover. IV. The influence of the host factors i_1 and *ie* upon nodule structure and cytology. *Heredity* 11, 175-184.

Beringer, J.E. (1984) The significance of symbiotic nitrogen fixation in plant production. *CRC Crit. Rev. Plant Sci.* 1, 269-286.

Bhagwat, A.A. and Thomas, J. (1982) Legume-*Rhizobium* interactions: cowpea root exudate elicits faster nodulation response by *Rhizobium* species. *Appl. Environ. Microbiol.* 43, 800-805.

Bhagwat, A.A. and Thomas, J. (1983) Legume-*Rhizobium* interactions: role of cowpea root exudate in polysaccharide synthesis and infectivity of *Rhizobium* species. *Arch. Microbiol.* 136, 102-105.

Bhagwat, A.A. and Thomas, J. (1984) Legume-*Rhizobium* interactions: host induced alterations in capsular polysaccharides and infectivity of cowpea *Rhizobium*. *Arch. Microbiol.* 140, 260-264.

Bhuvaneswari, T.V. (1984) Preinvasion events in Legume/*Rhizobium* symbiosis. In: C. Veeger and W.E. Newton, eds., Advances in Nitrogen Fixation Research. Martinus Nijhoff Publ., Dordrecht, The Netherlands, pp. 389-396.

Bhuvaneswari, T.V., Bhagwat, A.A. and Bauer, W.D. (1981) Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. *Plant Physiol.* 68, 1144-1149.

Bhuvaneswari, T.V., Mills, K.K., Crist, D.K., Evans, W.R. and Bauer, W.D. (1983) Effects of culture age on symbiotic infectivity of *Rhizobium japonicum* . *J. Bacteriol.* 153, 443-451.

Bhuvaneswari, T. V., Turgeon, B. G. and Bauer, W. D. (1980) Early events in the infection of soybean (*Glycine max* L. Merr.) by *Rhizobium japonicum*. I. Location of infectible root cells. *Plant Physiol.* 66, 1027-1031.

Bieberdorf, F.W. (1938) The cytology and histology of root nodules of some Leguminosae. *J. Am. Soc. Agron.* 30, 375-389.

Bohlool, B.B. and Schmidt, E.L. (1974) Lectins: a possible basis for specificity in the *Rhizobium* -Legume root nodule symbiosis. *Science* 185, 269-271.

Bojsen, K., Sandal, N. N. and Marcker, K. A. (1985) Structure and transcription of the soybean leghemoglobin and nodulin genes. In: H. J. Evans, P. J. Bottomley and W. E. Newton, eds., Nitrogen Fixation Research Progress, Martinus Nijhoff Publ.,Dordrecht, The Netherlands, pp. 47-52.

Bond, L. (1948) Origin and developmental morphology of root nodules of *Pisum sativum*. *Bot. Gaz.* 109, 411-434.

Bowra, B.J. and Dilworth, M.J. (1981) Motility and chemotaxis towards sugars in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* 126, 231-235.

Brockwell, J., Diatloff, A., Grassia, A. and Robinson, A.C. (1975) Use of wild soybean (*Glycine ussuriensis* Regel and Maack) as a test plant in dilution-nodulation frequency tests for counting *Rhizobium japonicum*. *Soil Biol. Biochem.* 7, 305-311.

Brockwell, J., Gault, R.R., Chase, D.L., Turner, G.L. and Bergersen, F.J. (1985) Establishment and expression of soybean symbiosis in a soil previously free of *Rhizobium japonicum*. *Aust. J. Agric. Res.* 36, 397-409.

Burnet, F.M. (1971) Self-recognition in colonial marine forms and flowering plants in relation to evaluation of immunity. *Nature* 232, 230-235.

Caldwell, B. E. (1966) Inheritance of a strain-specific ineffective nodulation in soybean. *Crop Sci.* 6, 427-428.

Caldwell, B.E., Hinson, K. and Johnson, H.W. (1966) A strain-specific ineffective nodulation reaction in the soybean *Glycine max* L. Merrill. *Crop Sci.* 6, 495-496.

Callaham, D.A. and Torrey, J.G. (1981) The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. *Can. J. Bot.* 59, 1647-1664.

Calvert, H.E., Pence, M.K., Pierce, M., Malik, N.S.A. and Bauer, W.D. (1984) Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. *Can. J. Bot.* 62, 2375-2383.

Cannon, J.R., Corbett, N.H., Brockwell, J., Gibson, A.H. and McIntyre, G.A. (1967) Nodulation and growth of *Trifolium subteranneum* L. cv. Mount Barker in agar culture. *Aust. J. Biol. Sci.* 20, 285-295.

Carroll, B. J. and Gresshoff, P. M. (1986) Isolation and initial characterization of constitutive nitrate reductase-deficient mutants NR328 and NR345 of soybean *(Glycine max)*. *Plant Physiol.* 81, 572-576.

Carroll, B. J., McNeil, D. L. and Gresshoff, P. M. (1985 a) A supernodulation and nitrate tolerant symbiotic (*nts)* soybean mutant. *Plant Physiol.* 78, 34-40.

Carroll, B. J., McNeil, D. L. and Gresshoff, P. M. (1985 b) Isolation and properties of soybean [*Glycine max* (L.) Merr.] mutants that nodulate in the presence of high nitrate concentrations. *Proc. Natl. Acad. Sci.* USA. 82, 4162-4166.

Carroll, B. J., McNeil, D. L. and Gresshoff, P. M. (1986) Mutagenesis of soybean [*Glycine max* (L.) Merr.] and the isolation of non-nodulating mutants. *Plant Sci.* 47, 109-114.

Chandler, M.R. (1978) Some observations on the infection of *Arachis hypogaea* L. by *Rhizobium*. *J. Exp. Bot.* 29, 749-755.

Chen, A.T. and Phillips, D.A. (1976) Attachment of *Rhizobium* to legume roots as the basis for specific interactions. *Physiol. Plant.* 38, 83-88.

Clark, F. E. (1957) Nodulation response of two near isogenic lines of the soybean. *Can. J. Microbiol.* 3, 113-123.

Crane, C. F., Beversdorf, W. D. and Bingham, E.T. (1982) Chromosome pairing and associations at meiosis in haploid soybean *(Glycine max)*. *Can. J. Genetic Cytol.*. 24, 293-300.

Currier, W.W. and Strobel, G.A. (1976) Chemotaxis of *Rhizobium spp.* to plant root exudates. *Plant Physiol.* 57, 820-823.

Damirgi, S.M., Fredrick, L.R. and Anderson, I.C. (1967) Serogroups of *Rhizobium japonicum* in soybean nodules as affected by soil types. *Agron. J.* 59, 10-12.

Dart, P.J. (1974) The infection process. In: A.Quispel, ed., The Biology of Nitrogen Fixation. North-Holland Publishing Company, Amsterdam, pp. 381-429.

Dart, P.J. (1977) Infection and development of leguminous nodules. In: R.W.F. Hardy, and W.S. Silver, eds., A Treatise on Dinitrogen Fixation. Section III. Biology. John Wiley and Sons, New York, pp. 367-472.

Dart, P.J. (1984) Application of biological nitrogen fixation for increasing crop production. In: C. Veeger and W.E. Newton, eds., Advances in Nitrogen Fixation Research. Martinus Nijhoff Publ., Dordrect, The Netherlands, p. 45.

Davis, T. M. (1985) Host genes affecting root nodule formation and function in chickpea *(Cicer arietinum* L.). In: H.J. Evans, P.J. Bottomley and W.E. Newton, eds., Nitrogen Fixation Research Progress. Martinus Nijhoff Publ., Dordrecht, The Netherlands, p. 40.

Davis, T. M., Foster, K. W. and Phillips, D. A. (1985) Nodulation mutants in chickpea. *Crop Sci.* 25, 345-348.

Davis, T. M., Foster, K. W. and Phillips, D. A. (1986) Inheritance and expression of three genes controlling root nodule formation in chickpea. *Crop Sci.* 26, 719-723.

Day, P. R., Barrett, J. A. and Wolfe, M. S. (1983) The evolution of host-parasite interations. In: T. Kosuge, C. Meredith and A. Hollaender, eds., Genetic Engineering of Plants. An agricultural perspective. Plenum Press, New York. pp. 419-430.

Day, D. A., Lambers, H., Bateman, J., Carroll, B. J. and Gresshoff, P. M. (1986) Growth comparisions of a supernodulating soybean *(Glycine max)* mutant and its wild-type parent. *Physiol. Plant.*. 68, 375-382.

Day, D. A., Price, G. D., Schuller, K. A. and Gresshoff, P.M. (1987) Nodule physiology of a supernodulating soybean *(Glycine max)* mutant. *Aust. J. Plant Physiol.* 14, 527-538.

Dazzo, F.B. and Brill, W.J. (1977) Receptor site on clover and alfalfa roots for *Rhizobium*. *Appl. Environ. Microbiol.* 33, 132-136.

Dazzo, F.B. and Hubbell, D.H. (1982) Control of root hair infection. In: W.J. Broughton, ed., Nitrogen Fixation, vol. 2, *Rhizobium*. Clarendon Press, Oxford, pp. 274-310.

Dazzo, F.B., Napoli, C.A. and Hubbell, D.H. (1976) Adsorption of bacteria to roots as related to host-specificity in the *Rhizobium* -clover symbiosis. *Appl. Environ. Microbiol.* 32, 166-171.

Dazzo, F.B. and Truchet, G.L. (1983) Interactions of lectins and their saccharide receptors in the *Rhizobium* -legume symbiosis. *J. Membrane Biol.* 73, 1-16.

Dazzo, F.B., Truchet, G.L., Sherwood, J.E., Hrabak, E.M., Abe, M. and Pankratz, H.S. (1984) Specific phases of root hair attachment in the *Rhizobium trifolii* -clover symbiosis. *Appl. Environ. Microbiol.* 48, 1140-1150.

Dazzo, F.B., Urbano, M.R. and Brill, W.J. (1979) Transient appearance of lectin receptors on *Rhizobium trifolii*. *Curr. Microbiol.* 2, 15-20.

Degenhardt, T. L., LaRue, T. A. and Paul, E. A. (1976) Investigation of a non-nodulating cultivar of *Pisum sativum*. *Can. J. Bot.* 54, 1633-1636.

Delves, A.C., Higgins, A.V. and Gresshoff, P.M. (1987) Shoot control of supernodulation in a number of mutant soybeans *Glycine max* (L.) Merr. *Aust. J. Plant Physiol.* 14, 689-694.

Delves, A. C., Mathews, A., Day, D. A., Carter, A. S., Carroll, B. J. and Gresshoff, P. M. (1986) Regulation of the soybean -*Rhizobium* symbiosis by shoot and root factors. *Plant Physiol.* 82, 588-590.

Devine, T. E. (1984). Inheritance of soybean nodulation response with a fast-growing strain of *Rhizobium*. *J. Heredity* 75, 359-361.

Devine, T. E. and Breithaupt, B. H. (1980) Significance of incompatibility reactions of *Rhizobium japonicum* strains with soybean host genotypes. *Crop Sci.* 20, 269-271.

Devine, T.E., Kuykendall, L.D. and Breithaupt, B.H. (1983a) Nodule-like structures induced on peanut by chlorosis producing strains of *Rhizobium* classified as *R. japonicum*. *Crop Sci.* 23, 394-397.

Devine, T. E., Palmer, R. G. and Buzzell, R. I. (1983b) Analysis of genetic linkage in the soybean. *J. Heredity* 74, 457-460.

Devine, T.E. and Weber, D.F. (1977) Genetic specificity of nodulation. *Euphytica* 26, 527-535.

Dowling, D.N. and Broughton, W.J. (1986) Competition for nodulation of legumes. *Ann. Rev. Microbiol.* 40, 131-157.

Downie, J.A., Ma, Q. -S., Knight, C.D., Rosen, L., Hombrecher, G., Brewin, N.J. and Johnston, A.W.B. (1984) Molecular genetics of nodulation and nitrogen fixation. *Proc. Eur. Phytochem. Soc.* 23, 61-87.

Dudley, M.E., Jacobs, T.W. and Long, S.R. (1987) Microscopic studies of cell divisions induced in alfalfa roots by *Rhizobium meliloti*. *Planta* 171, 289-301.

Dullaart, J. (1970) The bioproduction of indole-3-acetic acid and related compounds in root nodules and roots of *Lupinus luteus* L. and by its rhizobial symbiont. *Acta. Bot. Neerl.* 19, 573-615.

Dullaart, J. and Duba, L.T. (1970) Presence of gibberellin-like substances and their possible role in auxin bioproduction in root nodules and roots of *Lupinus luteus* L. *Acta Bot. Neerl.* 19, 877-883.

DuTeau, N.M., Palmer, R.G. and Atherly, A.G. (1986) Fast-growing *Rhizobium fredii* are poor nitrogen-fixing symbionts of soybean. *Crop Sci.* 26, 884-889.

Elkan, G.H. (1961) A nodulation-inhibiting root excretion from a non-nodulating soybean strain. *Can. J. Microbiol.* 7, 851-856.

Elkan, G.H. (1962) Comparison of rhizosphere microorganisms of genetically related nodulating and non-nodulating soybean lines. *Can. J. Microbiol.* 8, 79-87.

Eskew, D. L. and Schrader, L. E. (1977) Effect of rj_1rj_1 (non-nodulating) soybeans on nodulation of near isogenic Rj_1Rj_1 plants in nutrient culture. *Can. J. Microbiol.* 23, 988-993.

Fahraeus, G. (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J. Gen. Microbiol.* 16, 374-381.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C. and Signer, E.R. (1985) Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. *Cell* 40, 869-877.

Firmin, J. L., Wilson, K. E., Rossen, L. and Johnston, A. W. B. (1986) Flavanoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. *Nature* 324, 90-92.

Fisher, R.A. and Yates, F. (1963) Statistical Tables for Biological, Agricultural and Medical Research. Oliver and Boyd. London.

Gamborg, O. L. and Eveleigh, D. (1968) Detection of gluconases in suspension cultures of barley. *Can. J. Biochem.* 46, 417-422.

Gaworzewska, E.T. and Carlile, M.J. (1982) Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J. Gen. Microbiol.* 128, 1179-1188.

Gelin, O. and Blixt, S. (1964) Root nodulation in peas. *Agri. Hortique Genetica* 22, 149-159.

Gibson, A. H. (1963) Physical environment and symbiotic nitrogen fixation. I. The effect of root temperature on recently nodulated *Trifolium subterraneum* L. plants. *Aust. J. Biol. Sci.* 16, 28-42.

Goodchild, D.J. and Bergersen, F.J. (1966) Electron microscopy of the infection and subsequent development of soybean nodule cells. *J. Bacteriol.* 92, 204-213.

Gorbet, D. W. and Burton, J. C. (1979) A non-nodulating peanut. *Crop Sci.* 19, 727-728.

Graham, P. H. (1963) Antibiotic sensitivities of the root nodule bacteria. *Aust. J. Biol. Sci.* 16, 557-559.

Gresshoff, P.M. (1980) *In vitro* culture of white clover: callus suspension, protoplast culture and plant regeneration. *Bo. Gaz.* 141, 157-164.

Gresshoff, P. M. and Delves, A. C. (1986) Plant genetic approaches to symbiotic nodulation and nitrogen fixation in legumes. In: A. D. Blonstein, and P. J. King, eds., Plant Gene Research Vol. 3, Springer Verlag, Wien, pp. 159-206.

Halverson, L.J. and Stacey, G. (1984a) Host recognition in the *Rhizobium*-soybean symbiosis. Detection of a protein factor in soybean root exudate which is involved in the nodulation process. *Plant Physiol.* 74, 84-89.

Halverson, L.J. and Stacey, G. (1984b) Phenotypic reversal of a mutation in *Rhizobium japonicum* defective in nodule initiation. In: C.Veeger and W. Newton, eds., Advances in Nitrogen Fixation Research. Martinus Nijhoff Publ., Dordrecht, The Netherlands, p. 418.

Halverson, L.J. and Stacey, G. (1985) Host recognition in the *Rhizobium*-soybean symbiosis. Evidence for the involvement of lectin in nodulation. *Plant physiol.* 77, 621-625.

Halverson, L.J. and Stacey, G. (1986) Signal exchange in plant-microbe interactions. *Microbiol. Rev.* 50, 193-225.

Harper, J. E. (1974) Soil and symbiotic nitrogen requirements for optimum soybean production. *Crop Sci..* 14, 255-260.

Harper, J.E. (1976). Contributions of dinitrogen and soil or fertilizer nitrogen to soybean (*Glycine max* L. Merr.) production. In: L.D. Hill, ed., World Soybean Research. Interstate Printers and Publishers, Danville, Illinois pp. 101-107.

Harper, J.E., Nelson, R. S. and Streit, L. (1985) Nitrate metabolism of soybean - physiology and genetics. In: R. M. Shibles, ed., World Soybean Research Conference. III. Proceedings. Westview Press, Boulder, Colorado, pp. 476-483.

Hartman, T. and Glombitza, K.W. (1967) Der tryptophanabbau bei *Rhizobium leguminosarum*. *Arch. Mikrobiol.* 56, 1-8 (cited after Libbenga *et al.*, 1973).

Hely, F. W., Bonnier, C. and Manil, P. (1953) Effect of grafting on nodulation of *Trifolium ambiguum*. *Nature* 17, 884-885.

Hely, F.W. and Brockwell, J. (1962) An exploratory survey of the ecology of *Rhizobium meliloti* in inland New South Wales and Queensland. *Aust. J. Agric. Res.* 13, 864-879.

Herridge, D.F. (1977) Carbon and nitrogen nutrition of two annual legumes, Ph.D. Dissertation, University of Western Australia, Perth, Australia.

Higashi, S. and Abe, M. (1980) Promotion of infection thread formation by substances from *Rhizobium* . *Appl. Environ. Microbiol.* 39, 297-301.

Hirsch, A.M., Drake, D., Jacobs, T.W. and Long, S.R. (1985) Nodules are induced on alfalfa roots by *Agrobacterium tumefaciens* and *Rhizobium trifolii* containing small segments of the *Rhizobium meliloti* nodulation region. *J. Bacteriol.* 161, 223-230.

Hirsch, A.M., Wilson, K.J., Jones, J.D.C., Bang, M., Walker, V.V. and Ausubel, F.M. (1984) *Rhizobium meliloti* nodulation genes allow *Agrobacterium tumefaciens* and *Escherichia coli* to form pseudonodules on alfalfa. *J. Bacteriol.* 158, 1133-1143.

Holl, F.B. (1975) Host plant control of the inheritance of dinitrogen fixation in the *Pisum-Rhizobium* symbiosis. *Euphytica* 24, 767-770.

Hubbell, D.H. (1970) Studies on the root hair curling factor of *Rhizobium*. *Bot. Gaz.* 131, 337-342.

Hunter, W.J. and Fahring, C.J. (1980) Movement of *Rhizobium* and nodulation of legumes. *Soil Biol. Biochem.* 12, 537-542.

Jacobsen, E. and Feenstra, W. J. (1984) A new pea mutant with efficient nodulation in the presence of nitrate. *Plant Sci. Lett.* 33, 337-344.

Jacobsen, E., Postma, J.G., Nijdam, H. and Feenstra, W.J. (1985) Genetical and grafting experiments with pea mutants in studies on symbiosis. In: H.J.Evans, P.J. Bottomley and W.E.Newton, eds., Nitrogen Fixation Research Progress, Martinus Nijhoff Publ., Dordrecht, The Netherlands, p. 43.

Jensen, H. L. (1942) Nitrogen fixation in leguminous plants. I. General characteristics of the root nodule bacteria isolated from species of *Medicago* and *Trifolium*. In: Australian Proc. Linn. Soc. 66, 98-108.

Jensen, W.A. (1962) Botanical Histochemistry. W.H. Freeman and Co., San Francisco.

Jordan, D.C. (1984) Section 4. Gram-negative aerobic rods and cocci: Family III. Rhizobiaceae: Genus II. *Bradyrhizobium*. In: Bergey's Manual of Systematic Bacteriology Vol.1, Williams and Wilkins, Baltimore, pp. 234-256.

Katznelson, H. and Cole, S.E. (1965) Production of gibberellin-like substances by bacteria and actinomycetes. *Can. J. Microbiol.* 11, 733-741.

Kefford, N.P., Brockwell, J., Zwar, J.A. (1960) The symbiotic synthesis of auxin by legumes and nodule bacteria and its role in nodule development. *Aust. J. Biol. Sci.* 13, 456-567.

Kneen, B. E. and LaRue, T. A. (1984a) Nodulation resistant mutant of *Pisum sativum* (L.). *J. Heredity* 75, 238-240.

Kneen, B. E. and LaRue, T.A. (1984b) Peas *(Pisum sativum* L.*)* with strain specificity for *Rhizobium leguminosarum*. *Heredity* 52, 383-389.

Kneen, B. E., Vam Vikites, D. and LaRue, T. A. (1986) Nodulation mutants of *Pisum sativum*. In: D.P.S. Verma and N. Brisson, eds., Molecular Genetics of Plant-Microbe Interactions. Martinus Nijhoff Publ., Dordrecht, The Netherlands. pp. 79-84.

Kosslak, R. M. and Bohlool, B. B. (1984) Suppression of nodule development of the one side of a split root system of soybeans caused by prior inoculation of the other side. *Plant Physiol.*. 75, 125-130.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E. and Appelbaum, E. R. (1987) Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. 84, 7428-7432.

La Favre, J. S. and Eaglesham, A. R. J. (1984) Increased nodulation of "non-nodulating" (rj_1 rj_1) soybeans by high dose inoculation. *Plant Soil* 80, 297-300.

LaRue, T.A. (1980) Host plant genetics and enhancing symbiotic nitrogen fixation. In: W.D.P. Stewart and J.R. Gallon, eds., Nitrogen Fixation. Academic Press, London, pp. 355-364.

Law, I.J. and Strijdom, B.W. (1977) Some observations on plant lectins and *Rhizobium* specificity. *Soil Biol. Biochem.* 9, 79-84.

Law, I.R. and Strijdom, B.W. (1984) Role of lectins in the specific recognition of *Rhizobium* by *Lotononis bainesii*. *Plant Physiol.* 74, 779-785.

Lawn, R. J. and Brun, W. A. (1974) Symbiotic nitrogen fixation in soybean. III. Effect of supplemental nitrogen and intervarietal grafting. *Crop Sci.*. 14, 22-25.

Lawn, R. J. and Bushby, H. V. A. (1982) Effect of root, shoot and *Rhizobium* strain on nitrogen fixation in four Asiatic *Vigna* species. *New Phytol.* 92, 425-434.

Libbenga, K. R. and Harkes, P. A. A. (1973) Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. *Planta* 114, 17-28.

Libbenga, K. R., van Iren, F., Bogers, R. J. and Schraag-Lamers, M. F. (1973). The role of hormones and gradients in the initial of cortex proliferations and nodule formation in *Pisum sativum* L. *Planta* 114, 29-39.

Lie, T. A. (1971) Temperature-dependent root-nodule formation in pea cv. Iran. *Plant Soil* 34, 751-752.

Lie, T. A. (1984) Host genes in *Pisum sativum* conferring resistance to European *Rhizobium leguminosarum* . *Plant Soil* 82, 415-425.

Lie, T.A. and Timmermans, P.C.J.M. (1983). Host genetic control of nitrogen fixation in the legume-*Rhizobium* symbiosis: complication in the genetic analysis due to maternal effects. *Plant Soil* 75: 449-453.

Ljunggren, H. (1969) Mechanism and pattern of *Rhizobium* invasion into leguminous root hairs. *Physiol Plant. Suppl. V.*

McCoy, E.F. (1932) Infection by *Bact. radicola* in relation to the microchemistry of the host cell walls. *Proc. Roy. Soc. Ser. B.* 110, 514-533.

Malik, N.S.A., Calvert, H.E. and Bauer, W.D. (1987) Nitrate induced regulation of nodule formation in soybean. *Plant Physiol.* 84, 266-271.

Mathews, A., Carroll, B. J. and Gresshoff, P. M. (1987a) Characterization of non-nodulation mutants of soybean [*Glycine max* (L.) Merr.]: *Bradyrhizobium* effects and the absence of root hair curling. *J. Plant Physiol.* 131, 349-361.

Mathews, A., Carroll, B. J. and Gresshoff, P. M. (1987b) Non-nodulation mutants of soybean. In: D. P. S. Verma and N. Brisson, eds., Molecular Genetics of Plant-Microbe Interactions. Martinus Nijhoff Publ., Dordrecht, The Netherlands. pp. 94-95.

Mathews, A., Kosslak, R.M., Sengupta-Gopalan, C., Appelbaum, E.R., Carroll, B.J. and Gresshoff, P.M. Soybean *(Glycine max)* non-nodulation mutants have wild-type exudates and extracts. (in preparation).

Matthysse, A.G. (1983) The use of tissue culture in the study of crown gall and other bacterial diseases. In: J.P. Helgeson and D.I. Deverall, eds., Use of tissue culture and protoplasts in plant pathology. Academic Press Inc., New York, pp. 39-68.

Merril, C.R., Switzer, R.C. and Keuren, M.L. (1979) Trace polypeptides in cellular extracts and human body fluids detected by two dimensional electrophoresis and a highly sensitive silver stain. *Pro. Natl. Acad. Sci.* 76, 4335-4339.

Miller, J.H. (1972) Appendix I.Formulas and Recipes. In: Experiments in Molecular Genetics. Cold Spring Harbour Laboratory, New York.pp.433.

Mills, K.K. and Bauer, W.D. (1985) *Rhizobium* attachment to clover roots. *J. Cell Sci. Suppl.* 2, 333-345.

Moawad, A,A,, Ellis, W.R. and Schmidt, E.L. (1984) Rhizosphere response as a factor in competition among 3 serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. *Appl. Environ. Microbiol.* 47, 607-612.

Munns, D.N. (1968a) Nodulation of *Medicago sativa* in solution culture. II Compensating effects of nitrate and of prior nodulation. *Plant Soil* 28, 256-257.

Munns, D.N. (1968b) Nodulation of *Medicago sativa* in solution culture. III Effects of nitrate on root hairs and infection. *Plant Soil* 29, 33-47.

Munns, D.N. (1968c) Nodulation of *Medicago sativa* in solution culture. IV Effects of indole-3-acetate in relation to acidity and nitrate. *Plant Soil* 29, 257-262.

Napoli, C.A. and Hubbell, D.H. (1975) Ultrastructure of *Rhizobium*-induced infection threads in clover root hairs. *Appl. Microbiol.* 30, 1003-1009.

Newcomb, W. (1981) Nodule morphogenesis and differentiation. In: K.L. Giles and A.G. Atherly, eds., Biology of the Rhizobiaceae. Int. Rev. Cytol. Suppl. 13, Academic Press, New York, pp. 247-289.

Newcomb, W., Sippel, D. and Peterson, R.L. (1979) The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. *Can. J. Bot.* 57, 2603-2616.

Nigam, S. N., Arunachalam, V., Gibbons, R. W., Bandyopadhyay, A. and Nambiar, P. T. C. (1980) Genetics of nodulation in groundnut (*Arachis hypogaea* L.) *Oleagineux* 35, 453-455.

Nutman, P. S. (1949) Nuclear and cytoplasmic inheritance of resistance to infection by nodule bacteria in red clover. *Heredity* 3, 263-291.

Nutman, P.S. (1952) Studies on the physiology of nodule formation. III. Experiments on the excision of root-tips and nodules. *Ann. Bot.* 16, 79-101.

Nutman, P. S. (1954) Symbiotic effectiveness in nodulated red clover. II. A major gene for ineffectiveness in the host. *Heredity* 8, 47-60.

Nutman, P. S. (1957) Symbiotic effectiveness in nodulated red clover. III. Further studies on inheritance of ineffectiveness in the host. *Heredity* 11,157-173.

Nutman, P.S. (1959) Some observations on root hair infection by nodule bacteria. *J. Exp. Bot.* 10, 250-263.

Nutman, P.S. (1962) The relation between root hair infection by *Rhizobium* and nodulation in *Trifolium* and *Vicia*. *Proc. Roy. Soc.* London, 156, 122-137.

Nutman, P. S. (1968) Symbiotic effectiveness in nodulated red clover. V. The *n* and *d* factors for ineffectiveness. *Heredity* 23, 537-551.

Ohlendorf, H. (1985) Kontrollmechanismen der Leguminosen-*Rhizobium* Symbiose im Zusammenhang mit dem Infektionsprozess. *Angew Bot. 59,* 279-303.

Pappas, P.W. (1971) The use of chrome alum-gelatin (subbing) solution as a general adhesive for paraffin sections. *Stain Technol.* 46, 121-124.

Pate, J.S. (1958) Studies of the growth substances of legume nodules using paper chromatography. *Aust. J. Biol. Sci.* 11, 516-528.

Payne, J.H. and Pueppke, S.G. (1985) Rj_1 and rj_1 soybean isolines: adsorption of rhizobia to roots and distribution of primary root nodules. *Symbiosis* 1, 163-173.

Peters, K. N., Frost, J. W. and Long, S. R. (1986) A plant flavone, luteolin, indices expression of *Rhizobium meliloti* nodulation genes. *Science* 233, 977-980.

Peterson, M. A. and Barnes, D. K. (1981) Inheritance of ineffective nodulation and non-nodulation traits in alfalfa. *Crop Sci.*. 21, 611-616.

Phillips, D. A. (1971a) Abscisic acid inhibition of root nodule initiation in *Pisum sativum* L. *Planta* 100, 181-190.

Phillips, D.A. (1971b) A cotyledonary inhibitor of root nodulation in *Pisum sativum* . *Physiol. Plant.* 25, 482-487.

Phillips, D.A. and Torrey, J.G. (1970) Cytokinin production by *Rhizobium japonicum*. *Physiol. Plant.* 23, 1057-1063.

Pierce, M. and Bauer, W. D. (1983) A rapid regultory response governing nodulation in soybean. *Plant Physiol.* 73, 286-290.

Pueppke, S.G. (1983) *Rhizobium* infection threads in roots of *Glycine max* (L.) Merr., *Glycine soja* Sieb. and Zucc., and *Vigna unguiculata* (L.) Walp. *Can. J. Microbiol.* 29, 69-76.

Pueppke, S.G. (1984) Adsorption of slow-and fast-growing rhizobia to soybean and cowpea roots. *Plant Physiol.* 75, 924-928.

Pueppke, S.G. (1986) Physiology of nodule initiation in the nitrogen-fixing legume-*Rhizobium* symbiosis. *Physiol. Plant.* 67, 262-266.

Pueppke, S.G. and Payne, J.H. (1987) Responses of Rj_1 and rj_1 soybean isolines to inoculation with *Bradyrhizobium japonicum*. *Plant physiol.* 84, 1291-1295.

Radley, M. (1961) Gibberellin-like substances in plants. *Nature* 191, 684-685.

Ralston, E.J. and Imsande, J. (1983) Nodulation of hydroponically grown soybean plants and inhibition of nodule development by nitrate. *J. Exp. Bot.* 34, 1371-1378.

Rao, V.R. and Keister, D.L. (1978) Infection threads in the root hairs of soybean (*Glycine max*) plants inoculated with *Rhizobium japonicum*. *Protoplasma* 97, 311-316.

Redei, G.P. (1982) Modified Mendelian ratios. In: Genetics. Macmillan Publishing Company, New York, pp. 104-108.

Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L. and Rolfe, B. G. (1986) Flavones induce expression of nodulation genes in *Rhizobium*. *Nature* 323, 632-635.

Ridge, R.W. and Rolfe, B.G. (1986) Sequence of events during the infection of the tropical legume *Macroptilium atropurpureum* Urb. by the broad-host-range, fast-growing *Rhizobium* ANU240. *J. Plant Physiol.* 122, 121-137.

Robertson, J.G. (1981) Preinfection and infection process in the legume - *Rhzobium* symbiosis. In: A.H. Gibson and W.E. Newton, eds., Current Perspectives in Nitrogen Fixation. pp. 280-291.

Robertson, J.G. and Lyttleton, P. (1982) Coated and smooth vesicles in the biogenesis of cell walls, plasma membranes, infection threads and peribacteroid membranes in root hairs and nodules of white clover. *J. Cell Sci.* 58, 63-78.

Robin, P., Streit, L., Campbell, W. H. and Harper, J. E. (1985) Immunochemical characterization of nitrate reductase forms from wild-type (cv. Williams) and nr_1 mutant soybean. *Plant Physiol.* 77, 232-236.

Sahlman, K. and Fahraeus, G. (1963) An electron microscope study of root-hair infection by Rhizobium. *J. Gen. Microbiol.* 33, 425-427.

Salisbury, F.B. and Ross, C.W. (1978) Mineral Nutrition. In: Plant Physiology. 2nd Edition. Wadsworth Publ. Co. Inc., Belmont, California. p. 81

Sargent, L., Huang, S.Z., Rolfe, B.G. and Djordjevic, M.A. (1987) Split-root assays using *Trifolium subterraneum* show that *Rhizobium* infection induces a systemic response that can inhibit nodulation of another invasive *Rhizobium* strain. *Appl. Environ. Microbiol.* 53, 1611-1619.

Schaede, R. (1940) Die knollchen der adventiven wasserwurzeln von Neptunia oleracea und ihre bakteriensymbiose. *Planta* 31, 1-21.

Seegers, R. and LaRue, T.A. (1985) Legume agglutinins that bind to *Rhizobium meliloti*. *J. Bacteriol.* 162, 784-789.

Sharpe, A. N. and Jackson, A.K. (1972) Stomaching: a new concept in bacteriological sample preparation. *Appl. Microbiol.* 24, 175-179.

Sherwood, J.E., Vasse, T.M., Dazzo, F.B. and Truchet, G.L. (1984) Development and trifoliin A-binding ability of the capsule of *Rhizobium trifolii*. *J. Bacteriol.* 159, 145-152.

Shimshick, E.J. and Hebert, R.R. (1978) Adsorption of rhizobia to cereal roots. *Biochem. Biophys. Res. Commun.* 84, 736-742.

Smith, G. R. and Knight, W. E. (1984) Inheritance of ineffective nodulation in crimson clover. *Crop Sci.* 24, 601-604.

Soby, S. and Bergman, K. (1983) Motility and chemotaxis of *Rhizobium meliloti* in soil. *Appl. Environ. Microbiol.* 46, 995-998.

Stacey, G., Paau, A.S. and Brill, W.J. (1980) Host recognition in the *Rhizobium*-soybean symbiosis. *Plant Physiol.* 66, 609-614.

Stacey, G., Paau, A.S., Noel, K.D., Maier, R.J., Silver, L.E. and Brill, W.J. (1982) Mutants of *Rhizobium japonicum* defective in nodulation. *Arch. Microbiol.* 132, 219-224.

Stacey, G., Pocratsky, L.A. and Puvanesarajah, V. (1984) Bacteriophage that can distinguish between wild-type *Rhizobium japonicum* and a non-nodulating mutant. *Appl. Environ. Microbiol.* 48, 68-72.

Streeter, J.G. (1981) Effect of nitrate in the rooting medium of carbohydrate composition of soybean nodules. *Plant Physiol.* 68, 840-844.

Tanner, J. W. and Anderson, I. C. (1963) Investigations of non-nodulating and nodulating soybean strains. *Can. J. Plant Sci.* 43, 542-546.

Thornton, H.G. (1936) The action of sodium nitrate upon the infection of lucerne root hairs by nodule bacteria. *Proc. Roy. Soc.* London, 119, 474-492.

Torrey, J.G. and Barrios, S. (1969) Cytological studies on rhizobial nodule initiation in *Pisum*. *Caryologia* 22, 47-62.

Truchet, G., Rosenberg, G., Vasse, J., Tulliot, J.-S, Camut, S. and Denrie, J. (1984) Transfer of *Rhizobium meliloti* sym. genes into *Agrobacterium tumefaciens*. Host-specific nodulation by atypical infection. *J. Bacteriol.* 157, 134-142.

Turgeon, B.G. and Bauer, W.D. (1982) Early events in the infection by *Rhizobium japonicum*. Time course and cytology of the initial infection process. *Can. J. Bot.* 60, 152-161.

Turgeon. B.G. and Bauer, W.D. (1985) Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum*. *Planta* 163, 328-349.

van Egeraat, A.W.S.M. (1975a) Exudation of ninhydrin-positive compounds by pea-seedling roots: a study of the sites of exudation and the composition of the exudate. *Plant Soil* 42, 37-47.

van Egeraat, A.W.S.M. (1975b) The possible role of homoserine in the development of *Rhizobium leguminosarum* in the rhizosphere of pea seedlings. *Plant Soil* 42, 381-386.

van Rensberg, H.J. and Strijdom, B.W. (1982) Root surface association in relation to nodulation of *Medicago sativa*. *Appl. Environ. Microbiol.* 44, 93-97.

Vance, C.P. (1983) *Rhizobium* infection and nodulation: A beneficial plant disease? *Ann. Rev. Microbiol.* 37, 399-424.

Vesper, S.J. and Bauer, W.D. (1985) Characterization of *Rhizobium* attachment to soybean roots. *Symbiosis* 1, 139-162.

Vest, G. (1970) Rj_3 - A gene conditioning ineffective nodulation in soybean. *Crop Sci.*. 10, 34-35.

Vest, G. and Caldwell, B. E. (1972) Rj_4 - A gene conditioning ineffective nodulation in soybean. *Crop Sci.* 12, 692-693.

Vigue, J.T., Harper, J.E., Hugeman, R.H. and Peters, D.B. (1977) Nodulation of soybean grown hydroponically on urea. *Crop Sci.* 17, 169-172.

Vincent, J. M. (1970) A Manual for the Practical Study of Root-nodule Bacteria. IBP Handbook, No. 15., Blackwell Scientific Publications, Oxford and Edinburgh.

Vincent, J.M. (1974) Root-nodule symbiosis with *Rhizobium*. In: A. Quispel, ed., The Biology of Nitrogen Fixation. North-Holland Publ. Company, Amsterdam, pp. 265-341.

Vincent, J.M. (1980) Factors controlling the legume-*Rhizobium* symbiosis. In: W.E. Newton and W.H. Orme-Johnson, eds., Nitrogen Fixation, Volume II. Symbiotic associations and Cyanobacteria. University Park Press, Baltimore, USA. pp. 103-129.

Vorhees, J.H. (1915) Variations in soybean inoculations. *J. Amer. Soc. Agron.* 7, 139-140.

Ward, H.M. (1887) On the tubercular swellings on the roots of *Vicia faba*. *Phil. Trans. Roy. Soc. Ser. B.* 178, 539.

Weber, C. R. (1966) Nodulating and non-nodulating soybean isolines: I. Agronomic and chemical attributes. *Agron. J.* 58, 43-46.

West, P.M. and Wilson, P.W. (1939a) Growth factor requirements of the root nodule bacteria. *J. Bacteriol.* 37, 161-185.

West, P.M. and Wilson, P.W. (1939b) Effect of biotin concentrates on growth of *Rhizobium* and related species. *J. Bacteriol.* 38, 110-111.

Williams, L. F. and Lynch, D. L. (1954) Inheritance of a non-nodulating character in the soybean. *Agron. J.* 46, 28-29.

Williams, P.M. and Sicardi de Mallorca, M. (1982) Abscisic acid and gibberellin-like substances in roots and root nodules of *Glycine max* . *Plant Soil* 65, 19-26.

Williams, P.M. and Sicardi de Mallorca, M. (1984) Effect of gibberellins and the growth retardant CCC on the nodulation of soya. *Plant Soil* 77, 53-60.

Wipf, L. and Cooper, D.C. (1940) Somatic doubling of chromosomes and nodular infection in certain *Leguminosae*. *Amer. J. Bot.* 27, 821-824.

Wong, P.P. (1980) Nitrate and carbohydrate effects on nodulation and nitrogen fixation (acetylene reduction) activity of lentils *(Lens esculenta* Moench). *Plant Physiol.* 66, 78-81.

Yao, P.Y. and Vincent, J.M. (1969) Host specificity in the root hair "curling factor" of *Rhizobium* spp. *Aust. J. Biol Sci.*, 22, 413-423.

Yao, P.Y. and Vincent, J.M. (1976) Factors responsible for the curling and branching of clover root hairs by rhizobium. *Plant Soil* 45, 1-16.

Young, J.P.W., Johnston, A.W.B. and Brewin, N.J. (1982) A search for peas *(Pisum sativum* L.) showing strain specificity for symbiotic *Rhizobium leguminosarum*. *Heredity* 48, 197-201.